Volume 44 July 1958 Number 7

Proceedings of the
National Academy of Sciences
of the United States of America

The Proceedings of the National Academy of Sciences

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Entered as second-class matter June 21, 1920 at the Post-office at Easton, Pennsylvania, under the Act of August 24, 1912. Accepted for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorized June 18, 1920.

PRINTED IN THE U. S. A.

THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
is published for
THE NATIONAL ACADEMY OF SCIENCES
by
THE UNIVERSITY OF CHICAGO PRESS

Proceedings of the

NATIONAL ACADEMY OF SCIENCES

Volume 44 · Number 7 · July 15, 1958

*ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. III. THE INCORPORATION OF PYRIMIDINE AND PURINE ANALOGUES INTO DEOXYRIBONUCLEIC ACID**

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Communicated May 26, 1958

The synthesis of DNA[1] by an enzyme from *Escherichia coli* requires the presence of the deoxynucleoside triphosphates of adenine, guanine, cytosine, and thymine, magnesium ions, and DNA.[2, 3, 4] The product contains the deoxynucleotides joined by typical 3′,5′-phosphodiester linkages and can be formed in amounts up to 20 times the initial quantity of DNA added to the reaction mixture.[2, 3] Physical measurements indicate that the properties of the synthetic product are, in all respects measured, characteristic of DNA isolated from biological sources; it has a highly ordered rigid structure and an average molecular weight of about 5 million.[5] The reaction is specific for *deoxy*nucleoside *tri*phosphates, is reversed by high concentrations of inorganic pyrophosphate, and may be formulated thus:

$$\left.\begin{array}{l} n\ \text{dAPPP} \\ \quad + \\ n\ \text{dGPPP} \\ \quad + \\ n\ \text{dCPPP} \\ \quad + \\ n\ \text{TPPP} \end{array}\right\} + \text{DNA} \rightleftharpoons \text{DNA} - \left[\begin{array}{l} \text{dAP} \\ \\ \text{dGP} \\ \\ \text{dCP} \\ \\ \text{TP} \end{array}\right]_n + 4(n)\text{PP}$$

In view of reports that the pyrimidine and purine analogues, 5-bromouracil and 8-azaguanine, are incorporated into DNA in vivo,[6–10] it was of interest to determine whether our enzyme system would also accept substrates with such "unnatural" bases. The studies to be reported show that several pyrimidine and purine analogues[11] (uracil, hypoxanthine, 5-bromouracil, 5-bromocytosine, and 5-methylcytosine) are incorporated enzymatically into DNA. In each case the analogue substitutes specifically for the base it closely resembles with respect to the hydrogen-bonding properties required in the DNA structure proposed by Watson and Crick[12, 13] (Fig. 1).

METHODS AND MATERIALS

The analytical procedures and the preparation of the deoxynucleoside triphosphates of adenine, guanine, thymine, and cytosine were carried out as reported elsewhere.[2, 3]

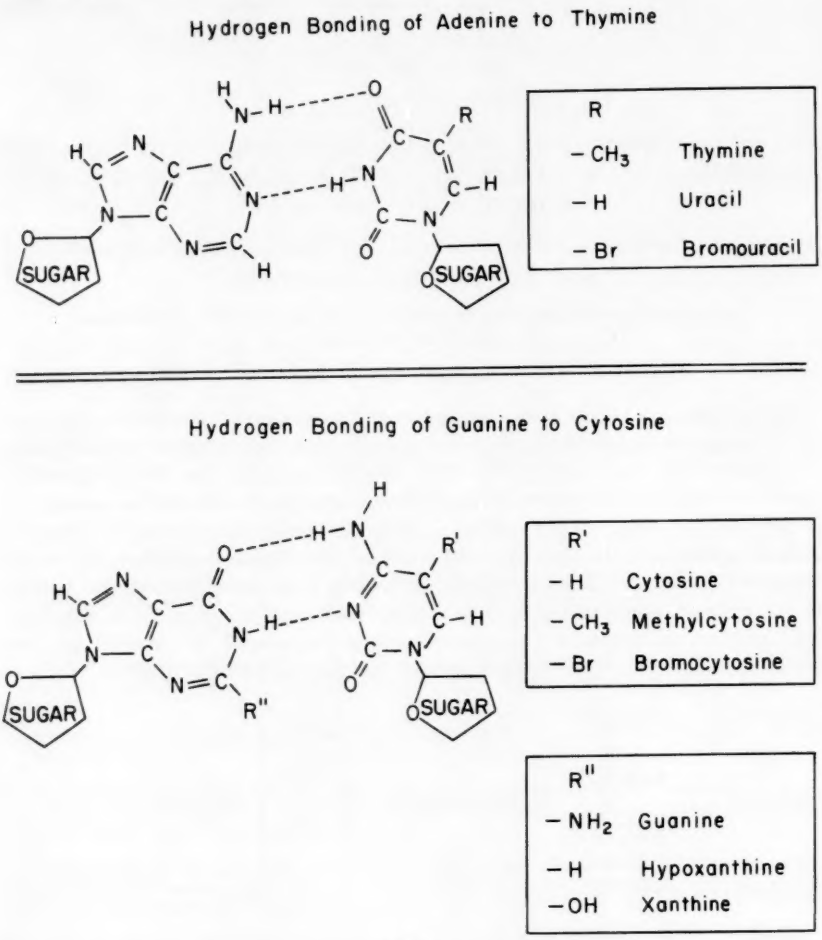

FIG. 1.

Assay for Deoxynucleotide Incorporation into DNA.—This is based on the conversion of a deoxynucleoside triphosphate (labeled with P^{32} in its innermost phosphate) to an acid-insoluble product. Complete details of this assay were provided elsewhere.[2]

Preparation of dUTP, dITP, and dXTP.—dUTP, dITP, and dXTP were prepared from dCTP, dATP, and dGTP, respectively by deamination with nitrous acid at 25° for 2 hours, according to Lohman.[14] The deaminated products were precipitated

as barium salts, converted to potassium salts by treatment with Dowex-50 K^+ at pH 5 at 0°, and purified by chromatography on Dowex-1 Cl columns.[2] The columns were washed first with enough 0.1 *M* LiCl–0.01 *N* HCl to elute completely the dCTP or dATP. dUTP or dITP was eluted in approximately 10 resin-bed volumes as a symmetrical peak by 0.20 *M* LiCl–0.02 *N* HCl and obtained in yields of approximately 60 per cent from 2 μmoles of starting material. dXTP was not chromatographed; spectrophotometric analysis after treatment with nitrous acid showed that at least 95 per cent of the dGTP had been deaminated.

Preparation of 5-Bromo dCTP and 5-Bromo dUTP.—dCTP was brominated according to the procedure of Markham,[15] by adding dropwise a solution of bromine in carbon tetrachloride to dCTP dissolved in formamide. The uptake of bromine was instantaneous; the addition was stopped when an excess had been added, as shown by persistence of a yellow color. Excess bromine was removed by adding a drop of aniline; the brominated dCTP was then precipitated as the barium salt, converted to the potassium salt, and purified by chromatography on paper.[16] 5-Bromo dUTP was prepared by deamination of 5-bromo dCTP in nitrous acid as described for the preparation of dUTP. It was purified by paper electrophoresis at pH 9.3 according to Dunn and Smith.[10]

Preparation of 5-Methyl dCTP.—5-Methyl deoxycytidylate was isolated from wheat-germ DNA according to Sinsheimer and Koerner,[17, 18] and was converted to the triphosphate by the procedure of Khorana.[19, 20] Analyses of all the analogues are reported in Table 1.

TABLE 1

ANALYTIC DATA FOR ANALOGUES OF THE NATURAL DEOXYNUCLEOSIDE TRIPHOSPHATES

Analysis	dUTP	5-Bromo dUTP	5-Methyl dCTP	5-Bromo dCTP	dITP	dXTPs
Total P (moles)	3.00	3.00	3.00	3.00	3.00	3.00
Acid-labile P (moles)*	1.85	1.93	1.86	2.18	...	...
Base (moles)	1.11	0.98	1.01	...	1.07	0.94
Deoxypentose (moles)	...	...	...	...	0.91	...
λ max ($m\mu$)†	262	279	286	299	249	263
λ 280/λ 260	0.45	1.77	3.00	4.0	0.27	0.32
λ 250/λ 260	0.72	0.62	0.46	1.10	1.62	0.72

* Determined as inorganic ortho P after 15 minutes in 1 *N* H_2SO_4 at 100°.
† Spectral measurements were at pH 1 except for dITP, which was at pH 7.

Enzymes.—Micrococcal DNase was kindly provided by Dr. Lew Cunningham. Spleen phosphodiesterase was obtained as a gift from Dr. Leon Heppel, and more was prepared according to the method of Hilmoe and Heppel.[21] Semen phosphomonoesterase was prepared according to Kornberg and Wittenberg.[22] The deoxynucleotide kinase(s) of *E. coli* was prepared as described previously.[2] The DNA-synthesizing enzyme from *E. coli* was prepared from the "polymerase" fraction VII described elsewhere[2] by refractionating with diethylaminoethylcellulose in the identical manner as fraction VII. It is referred to as "fraction VII-R."

RESULTS

Replacement of the "Natural" Bases of DNA by Their Analogues.—dUTP used in place of TTP supported DNA synthesis at 54 per cent of the rate of the control value but failed to support synthesis when used in place of dATP, dGTP, or dCTP

(Table 2). 5-Bromo-dUTP was more effective as a replacement for TTP but was unable to substitute for any of the other triphosphates. The 5-bromo and 5-methyl dCTP derivatives replaced only dCTP and were even more effective than dCTP itself in promoting DNA synthesis; evaluation of the significance of these higher rates requires further kinetic studies. dITP permitted a reduced rate of DNA synthesis in the absence of dGTP, but essentially no synthesis when any one of the other triphosphates was absent.

TABLE 2

REPLACEMENT OF NATURAL BASES BY ANALOGUES IN ENZYMATIC SYNTHESIS OF DNA

Expt. No.	Control Value* (mμ Moles)	Base Analogue Used	Natural Base Omitted (Per Cent of Control)†			
			Thymine	Adenine	Guanine	Cytosine
1	0.50	Uracil	**54**	4	6	
1*a*	0.88	Uracil				3
2	0.43	5-Bromouracil	**97**	2	4	
2*a*	0.42	5-Bromouracil				4
3	0.51	5-Bromocytosine		4	4	**118**
3*a*	0.40	5-Bromocytosine	4			
4	0.58	5-Methylcytosine		2	3	**185**
4*a*	0.52	5-Methylcytosine	2			
5	0.37	Hypoxanthine		3	**25**	5
5*a*	0.27	Hypoxanthine	4			

Control values are mμmoles of radioactive deoxynucleotide incorporated into DNA in the absence of analogue. Incubation mixtures contained in 0.3 ml., 5 mμmoles each of TTP, dATP, dCTP, and dGTP; 2 μmoles of $MgCl_2$; 20 μmoles of potassium phosphate (pH 7.4); 10 μg. of calf-thymus DNA; and 1 μg. of enzyme fraction VII-R. Experiments were performed at 37° for 30 minutes. Labeled substrates were: $dCP^{32}PP$ in Expts. 1, 2, 5*a*; $TP^{32}PP$ in Expts. 1*a*, 3, 4, 5; and $dGP^{32}PP$ in Expts. 2*a*, 3*a*, 4*a*.

† The percentage value represents the fraction of the labeled substrate incorporated when the analogue (5 mμmoles) was used instead of a natural base. All bases, natural or analogue, were supplied as the deoxynucleoside triphosphates. Values of 5 per cent or below are near the limit of detectability and are of questionable significance

TABLE 3

INCORPORATION OF P^{32}-DEOXYURIDYLATE INTO DNA

Substrates†	P^{32}-Deoxynucleotide Incorporated (mμMoles)
$\overset{*}{U} + T + C + A + G$	0.14
Omit T	0.38
Omit C	0.01
Omit A	0.01
Omit G	0.01
$U + \overset{*}{T} + C + A + G$	0.37
Omit U	0.77

† U = dUTP; $\overset{*}{U}$ = $dUP^{32}PP$; T = TTP; $\overset{*}{T}$ = $TP^{32}PP$; C = dCTP; A = dATP; G = dGTP.

Direct Demonstration of the Incorporation of Deoxyuridylate into DNA.—Deoxyuridylate was incorporated into DNA at about half the rate of thymidylate (Table 3, compare lines 2 and 7); omission of the triphosphates of deoxycytidine, deoxyadenosine, or deoxyguanosine practically eliminated the incorporation of deoxyuridylate. With $dIP^{32}PP$ comparable results were obtained, except that deoxyinosinate was incorporated into DNA specifically in the place of deoxyguanylate and at 25 per cent the rate. However, $dXP^{32}PP$ was not incorporated into DNA. Under the same conditions in which $dGP^{32}PP$ was incorporated to the extent of 0.66 mμmole, less than 0.02 mμmole of $dXP^{32}PP$ was incorporated into an acid-insoluble product. The possibility that the chemically synthesized dXTP contained an inhibitor was ruled out by the observation that dXTP added to the normal reaction mixture had no inhibitory effect.

Demonstration of Phosphodiester Linkages between Deoxyuridylate and the Other Deoxynucleotides in Enzymatically Synthesized DNA.—DNA containing radioactive deoxyuridylate was prepared (Table 4), mixed with a 50-fold excess of

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN DEOXYNUCLEOSIDE 3′-PHOSPHATES ISOLATED FROM ENZYMATICALLY SYNTHESIZED DNA CONTAINING DEOXYURIDINE 5′-P^{32}

Isolated 3′-Deoxynucleotides	Amount* (μMoles)	Radioactivity (c.p.m. × 10^{-3})
Cytidylate	0.17	2.0
Adenylate	0.25	2.3
Guanylate	0.22	1.7
Uridylate	...	2.5
Thymidylate	0.24	0.0

* The recovery of deoxynucleotides and P^{32} from the paper electrophoresis strip was 96 per cent.

The incubation mixture for the synthesis of deoxyuridylate-containing DNA included dCTP, dATP, dGTP (75 mμmoles of each), $dUP^{32}PP$ (75 mμmoles, 3.8 × 10^6 c.p.m./μmole), $MgCl_2$ (30 μmoles), potassium phosphate (300 μmoles, pH 7.4), calf-thymus DNA (300 μg.), enzyme (15 μg. of Fraction VII-R), and water to a final volume of 4.5ml. After 30 minutes at 37°, the reaction mixture was chilled and 2.5 mg. of calf-thymus DNA were added as "carrier." The DNA was precipitated by the addition of perchloric acid (final concentration, 0.5 *N*) and the precipitate was dissolved in dilute NaOH. The precipitation was repeated several times until the acidic supernatant fluid contained less than 1 per cent of the radioactivity of the precipitated DNA. Details of the enzymatic digestion of the DNA and the subsequent isolation of the deoxynucleoside 3′-phosphates are in the text.

TABLE 5

SPECIFICITY OF DEOXYNUCLEOTIDE KINASE(S) OF *E. coli* TOWARD VARIOUS DEOXYNUCLEOTIDES

Expt. No.	Conditions	P^{32}-Deoxynucleotide Incorporated into DNA: With Kinase (mμMoles)	Without Kinase (mμMoles)
1	Control*	0.74	0.74
	Omit TTP	0.04	...
	Omit TTP, add thymidylate	0.83	...
	Omit TTP, add 5-bromodeoxyuridylate	0.63	0.01
	Omit TTP, add deoxyuridylate	0.04	...
2	Control	0.49	0.50
	Omit dCTP	0.02	...
	Omit dCTP, add deoxycytidylate	0.50	...
	Omit dCTP, add 5-methyldeoxycytidylate	0.02	...

* Incubation mixture containing all four deoxynucleoside triphosphates as described in Table 2, and also 0.25 μmole of ATP. The phosphorylation of a deoxynucleotide is measured, as in line 3 for thymidylate, by the sequence of reactions:

$$\text{Thymidylate } (25\ \text{m}\mu\text{moles}) \xrightarrow[\text{ATP}]{36\ \mu\text{g. of } E.\ coli\ \text{kinase(s)}} \text{TTP} \xrightarrow[\text{dCTP/dGTP}]{\text{polymerizing enzyme Fraction VII-R; dAP}^{32}\text{PP}} \text{DNA-P}^{32}$$

The incorporation of $dAP^{32}PP$ is absolutely dependent on the formation of TTP from thymidylate. Phosphorylation of the other deoxynucleotides was tested under analogous conditions with the assumption that the corresponding triphosphate is in each case essential for DNA synthesis (see Table 2).

thymus DNA as "carrier," and digested first with micrococcal DNase and then with spleen phosphodiesterase. This treatment degrades the DNA to 3′-deoxynucleotides; the P^{32} of $dUP^{32}PP$ should therefore now be bound to the deoxynucleoside with which it formed phosphodiester linkages.[23] The hydrolysis with micrococcal DNase was allowed to proceed until 95 per cent of the radioactivity was converted to an acid-soluble form. Incubation with spleen phosphodiesterase rendered 90 per cent of the radioactivity sensitive to semen phosphomonoesterase. At this point, at least 80 per cent of the original DNA product had been converted

to deoxynucleoside 3′-phosphates. The preparation was desalted by adsorption on Norit and elution with ammoniacal ethanol and then subjected to electrophoresis as described by Markham and Smith[24] (Whatman 3-MM. paper, pH 3.5 in 0.02 *M* ammonium acetate buffer). The bands were eluted and identified by their spectral characteristics, and the eluates were assayed for radioactivity.

It can be seen in Table 4 that all four of the 3′-deoxynucleotide fractions were labeled with P^{32}. Thus P^{32} which originally entered the enzymatically synthesized DNA as deoxyuridylate had been in phosphodiester linkage with cytosine, adenine, guanine, and uracil deoxynucleosides. Since TTP was omitted from the reaction mixture, it might be expected that little or no radioactivity would be found in the isolated thymidine 3′-phosphate. Deoxyuridylate is not separated from thymidylate under these conditions of paper electrophoresis, and it was necessary to resolve these nucleotides by paper chromatography.[16] All the radioactivity was found in the deoxyuridylate area and less than 5 per cent was in the thymidylate region.[25]

Specificity of Deoxynucleotide Kinase(s) of E. coli toward Various Deoxynucleotides.—The data in Table 5 indicate, in confirmation of an earlier report,[26] that, while the naturally occurring deoxynucleotides such as thymidylate and deoxycytidylate are readily converted to the triphosphate level, the analogue deoxyuridylate is not. However, the result showing 5-bromo deoxyuridylate to be a favorable substrate for phosphorylation is consistent with interpretations from in vivo studies[27] that bromouracil is incorporated into DNA via a deoxynucleotide intermediate.

It is of interest to point out that 5-methyl deoxycytidylate is not converted to the triphosphate by extracts of *E. coli* (Table 5), although when supplied as the triphosphate it is incorporated into DNA (Table 2).

DISCUSSION

In the Watson and Crick model of DNA,[12, 13] two polynucleotide strands are coiled about a central axis and are held together by hydrogen bonds through specific pairings between thymine and adenine and between guanine and cytosine. Our data demonstrate that deoxyuridylate and 5-bromo deoxyuridylate are incorporated into enzymatically synthesized DNA and that they specifically replace thymidylate. In a like manner deoxycytidylate may be replaced by the 5-methyl or 5-bromo derivatives, and deoxyguanylate may be replaced by deoxyinosinate. Substitution of a hydrogen or a bromine atom for a methyl group in the 5-position of thymine still maintains the keto grouping in the 6-position and an available H in the 1-position for hydrogen-bonding with the 6-amino and 1-N groups, respectively, of adenine (Fig. 1). Since the methyl group in thymine plays no assigned role in the hydrogen-bonding of thymine to adenine, it is not surprising that the other uracil derivatives replace thymine so well in the enzymatic synthesis of DNA. The results obtained with the cytosine analogues may be regarded in a similar way. Although hypoxanthine lacks the amino group at the 2-position in guanine, the other two substituents responsible for the hydrogen-bonding of guanine to cytosine remain. It is possible that the substantially lower rate of deoxyinosinate incorporation into DNA as compared with deoxyguanylate may be due to the absence of the third hydrogen bond with the keto group of cytosine.[28] At present no definitive explanation can be given for the failure of deoxyxanthylate to be incorporated into DNA in place of deoxyguanylate.

The specific replacement of the purines and pyrimidines of DNA by analogues with similar hydrogen-bonding capacities is in agreement with the specific base pairing in the Watson and Crick model. However, it would be premature to state a priori that a given analogue will be incorporated into DNA simply from a consideration of its capacity to form specific hydrogen bonds with the bases in DNA. The specificity of the enzyme toward a given substrate must be considered, as well as the unexplored influence of the DNA primer. For these reasons it is not feasible at this time to attribute different rates of incorporation of dUTP, dITP, and the natural deoxynucleoside triphosphates to conditions controlled simply by hydrogen-bonding characteristics of base pairs.

It should be emphasized that the results in this paper are only approximations to reaction rates and do not provide any indication as to how the analogues support extensive net synthesis of DNA. Further studies of the reaction kinetics with the analogues are necessary, as are investigations of the primer capacity of the DNA containing the analogue.

Since deoxyuridylate replaces thymidylate so readily in the enzymatic synthesis of DNA, it is pertinent to inquire why uracil has never been observed as a component of DNA in nature. The answer appears to lie in an observation previously noted[26] and further documented here that there may not be any mechanism for phosphorylating deoxyuridylate to the triphosphate form. While high levels of kinase activity for the conversion of adenine, guanine, thymine, and cytosine deoxynucleotides to the respective triphosphates are evident in extracts of *E. coli*, we have not yet been able to demonstrate comparable activity toward deoxyuridylate. It is of further interest that 5-bromodeoxyuridylate is readily converted to the triphosphate by *E. coli* preparations and is thus consistent with the known capacity of *E. coli* cells to incorporate 5-bromouracil into its DNA.[9, 10]

SUMMARY

The deoxyribonucleoside triphosphates of analogues of the pyrimidine and purine bases were prepared chemically and tested as substrates for the enzyme which polymerizes deoxyribonucleotides to deoxyribonucleic acid.

Uracil and 5-bromouracil were incorporated into deoxyribonucleic acid specifically in place of thymine; 5-methyl- and 5-bromocytosine in place of cytosine; and hypoxanthine in place of guanine. Xanthine was not incorporated into deoxyribonucleic acid. Analysis of the uracil-containing product demonstrated that the uracil deoxyribotide was bound in 3′-5′ phosphodiester linkage with each of the deoxyribotides of adenine, guanine, cytosine, and uracil.

The specific replacement of the natural bases by these analogues offers additional support for the base-pairing relationships in the double helix proposed by Watson and Crick for the structure of deoxyribonucleic acid.

The existence of kinases in *E. coli* which phosphorylate 5-bromodeoxyuridylate to the triphosphate and the apparent absence of such kinases for the phosphorylation of deoxyuridylate are in keeping with the fact that cells incorporate 5-bromouracil, but not uracil, into DNA.

We are indebted to Dr. Roy Markham who while visiting our laboratory was very helpful in several aspects of this work.

* This investigation was supported by research grants from the National Institutes of Health, Public Health Service, and the National Science Foundation.

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[1] The abbreviations used in this report are: ATP, adenosine triphosphate; c.p.m., counts per minute; dATP or dAPPP, deoxyadenosine triphosphate; dCTP or dCPPP, deoxycytidine triphosphate; dGTP or dGPPP, deoxyguanosine triphosphate; TTP or TPPP, thymidine triphosphate; dITP or dIPPP, deoxyinosine triphosphate; dUTP or dUPPP, deoxyuridine triphosphate; dXTP or dXPPP, deoxyxanthosine triphosphate; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; PP, inorganic pyrophosphate.

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[16] The solvent (system No. III, described in circular No. OR-10 of the Pabst Laboratories) contained 600 gm. of ammonium sulfate, 1 liter of 1 *M* sodium phosphate (pH 6.8), and 20 ml. of *n*-propanol.

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[19] We are indebted to Dr. H. G. Khorana for sending us his procedure for the synthesis of deoxynucleoside triphosphates before it was submitted for publication.

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[22] J. Wittenberg and A. Kornberg, *J. Biol. Chem.*, **202**, 431, 1953.

[23] The use of micrococcal DNase and spleen phosphodiesterase to establish the existence of a phosphodiester linkage between adjacent deoxynucleotides in a polydeoxyribonucleotide is also illustrated in the following paper, where details of the procedure are given.

[24] R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552, 1952.

[25] A trace of P^{32}-thymidylate might be anticipated in this experiment. As shown in the succeeding paper, this would occur in every instance in which deoxyuridylate-P^{32} reacted with a strand of DNA terminating in a thymidine residue. The sensitivity of measurements in this experiment was not adequate to detect such traces of thymidine 3′-phosphate.

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ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. IV. LINKAGE OF SINGLE DEOXYNUCLEOTIDES TO THE DEOXYNUCLEOSIDE ENDS OF DEOXYRIBONUCLEIC ACID

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Communicated May 26, 1958

Although the synthesis of DNA[1] by an enzyme purified from *Escherichia coli* requires four deoxynucleoside triphosphates (dAPPP, dCPPP, dGPPP, TPPP) in addition to Mg^{++} and DNA,[2, 3] nevertheless a single deoxynucleoside triphosphate can be incorporated into DNA to an extent about one-thousandth of that for the complete reaction.[3] The number of the single deoxynucleotides incorporated is of the same order as an approximation of the number of DNA chains added to the reaction mixture.

It was the purpose of this investigation to determine whether the single deoxynucleotide, incorporated in the absence of the other three deoxynucleotide substrates, was covalently linked to the DNA and, if so, where in the DNA chain. It was also of interest to establish whether only one or several deoxynucleotides were added to a DNA chain. The results show that the single deoxynucleoside triphosphate reacts with the deoxynucleoside end of a DNA chain to form a typical 3′,5′-phosphodiester linkage and that only one or a few nucleotides are added to a chain.

METHODS AND MATERIALS

Materials.—Uniformly labeled C^{14}-5′-deoxycytidylate was obtained by enzymatic hydrolysis of DNA from a *Chromatium* species grown on $C^{14}O_2$ as the sole carbon source. Deoxyribonucleoside triphosphates were prepared as described previously.[2] Micrococcal DNase[4] was generously supplied by Dr. L. Cunningham. Bull semen 5′-nucleotidase[5] and purified human semen phosphomonoesterase were kindly provided by Dr. L. A. Heppel. Calf spleen phosphodiesterase was prepared according to Hilmoe and Heppel.[6] Venom phosphodiesterase was prepared from the venom of *Crotalus adamanteus* according to Koerner and Sinsheimer[7] and was free of phosphomonoesterase. The DNA-synthesizing enzyme used in these studies (Fraction VII) was purified about 2,000-fold.[2] Calf thymus DNA was prepared according to Kay, Simmons, and Dounce.[8]

Preparation of DNA products.—Four products were prepared from $dAP^{32}PP$, $dCP^{32}PP$, $TP^{32}PP$, or $C^{14}dCPPP$, essentially as described previously,[2] except that the concentration of DNA was raised 25-fold in order to obtain greater amounts of incorporation. The reaction mixtures contained (per ml.) 6.0 mμmoles of a deoxynucleoside triphosphate; 6.7 μmoles of magnesium chloride; 67 μmoles of potassium phosphate buffer at pH 7.5; 800 μg. of calf thymus DNA; and 20 μg. of Fraction VII enzyme. The volume of these reaction mixtures ranged from 18 to 36 ml. Incubations were carried out at 37° for 2.5 hours (for $dAP^{32}PP$, $dCP^{32}PP$, and $TP^{32}PP$) or for 1 hour (for $C^{14}dCPPP$).

The reactions were stopped and the products freed of unreacted deoxynucleoside triphosphate in one of two ways: (1) acidification with an equal volume of cold 1 *N* perchloric acid, then alternate solubilization of the precipitated product in 0.1 *N* NaOH and precipitation in 0.5 *N* perchloric acid (when enzyme had been omitted, no radioactivity could be detected in this repeatedly precipitated product); (2) addition of sodium chloride to a final concentration of 0.2 *M* and heating at 70° for 10 minutes,[3] then repeated dialysis against 0.2 *M* sodium chloride. The radioactivity in this dialyzed product was then entirely precipitable by perchloric acid.

RESULTS

Extent and Kinetics of Reaction.—In the four DNA products prepared, the incorporation of dAP^{32}, dCP^{32}, TP^{32}, and $C^{14}dCP$ was 0.25, 0.13, 0.32, and 0.34 mμmoles, respectively, per milliliter of reaction mixture. For an assumed molecular weight of 5×10^6 for thymus DNA, these amounts correspond to an incorporation of 0.5 to 1 mole of deoxynucleotide per mole of DNA. Several factors add uncertainty to this approximation: the polydispersity of DNA, lack of information about the size and number of competent DNA molecules, and the hydrolysis of DNA to smaller molecules during the incubation.

TABLE 1*

ULTRACENTRIFUGAL ANALYSIS OF PRODUCT

Expt. No.	Basis for Calculation	Fraction Remaining in Top Compartment (Per Cent)	Sedimentation Coefficient (*S*)
1	Schlieren boundary	..	12.3
	Optical density at 260 mμ	48	12.8
	Radioactivity	48	13.2
2	Schlieren boundary	..	12.7
	Optical density at 260 mμ	50	12.7
	Radioactivity	52	12.5
3†	Optical density at 260 mμ	10	22.1
	Radioactivity	13	21.4

* In these experiments the product used was from a reaction with $TP^{32}PP$ as the only deoxynucleoside triphosphate. The sedimentation coefficients were measured at a relatively high concentration (optical density at 260 mμ = 7.0) in order to achieve significant measurements of radioactivity. When the same product was run at a tenfold dilution, the sedimentation coefficient was 21 *S*.

† In experiment 3 the product had been heated at 100° for 10 minutes. As a result of the heating, the schlieren boundaries were too diffuse to serve as a basis for calculation of a sedimentation coefficient.

A study of the time course of the reaction between DNA and a single deoxynucleoside triphosphate showed an initial rapid rate of incorporation followed by a slower rate. In a typical experiment with $dCP^{32}PP$ as the only deoxynucleoside triphosphate, the incorporation into DNA at 30, 60, and 120 minutes was 4.0, 5.0, and 7.0 μμmoles, respectively. This slow rate of incorporation after the initial rapid reaction could be explained by the production of new, reactive DNA ends. Such degradation of the DNA, catalyzed by a contaminating nuclease, is indicated by a small decrease in viscosity during the preparation of the products. Another contaminating nuclease has the property ascribed to snake venom phosphodiesterase (see below) of removing deoxynucleotides from the deoxynucleoside ends of DNA chains.

Ultracentrifugal Analysis of the Product Showing the Incorporation of Deoxynucleotides into Molecules the Size of DNA.—In order to determine whether the incorporated radioactive deoxynucleotide possesses the same sedimentation rate as

the average DNA molecule, a product was examined in a fixed partition separation cell[9] in the ultracentrifuge. Centrifugation was stopped after about half of the DNA had sedimented out of the top compartment of the separation cell, as determined by the schlieren optical system. Then the fractions of the optical density and radioactivity remaining in the upper compartment of the cell were measured, and sedimentation coefficients were calculated from these data and from the schlieren boundaries.[10]

As shown in Table 1, the sedimentation coefficients determined from the three measurements agree. Even after the products had been heated at 100° for 10 minutes, to try to break down the structure of the macromolecules,[11] the radioactivity still sedimented at the same rate as the DNA. These data suggest that the radioactive deoxynucleotide had formed covalent linkages, rather than much weaker secondary bonds, to molecules of the same average size as DNA.

Hydrolysis of Products to 3′-Deoxynucleotides Demonstrating 3′,5′-Phosphodiester Linkages between the Incorporated Deoxynucleotides and Deoxynucleoside Ends of DNA Chains.—To determine whether the incorporated P^{32} deoxynucleotide is bound to DNA by a phosphodiester linkage, a method of hydrolysis was sought which would leave the P^{32} with the adjacent deoxynucleotide (Fig. 1). For RNA such a result has been accomplished by hydrolysis to 2′- and 3′-ribonucleotides with alkali.[12, 13] For DNA no such reagent was available until the recent discovery and purification of a DNase from *Micrococcus pyogenes*[4] and a phosphodiesterase from calf spleen.[6] The successive action of these two enzymes has now resulted in nearly complete hydrolysis of DNA to 3′-deoxynucleotides. Koerner and Sinsheimer have obtained a similar result by combining spleen DNase and spleen phosphodiesterase.[7]

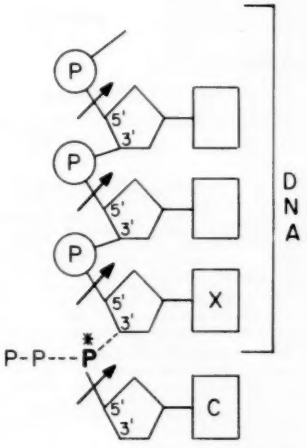

FIG. 1.—Hydrolysis of product to 3′-deoxynucleotides by the combined action of micrococcal DNase and spleen phosphodiesterase. $P^* = P^{32}$

In the present experiments the progress of hydrolysis was followed by measuring phosphate released by human semen phosphomonoesterase. Micrococcal DNase in 0.01 *M* $CaCl_2$ and 0.1 *M* tris(hydroxymethyl)aminomethane at pH 8.5 hydrolyzed the products to acid-soluble fragments containing 55–65 per cent of their total phosphate in the monoester form. Spleen phosphodiesterase then added to the digest (adjusted to pH 7.0 with HCl) converted 86–94 per cent of the total phosphate to the monoester level. No inorganic phosphate (<2 per cent of the total phosphate) could be detected at the end of the digestion. The resulting 3′-mononucleotides, separated by chromatography, were sensitive to semen phosphomonoesterase but were entirely resistant to 5′-nucleotidase under conditions where 5′-deoxynucleotides were completely hydrolyzed.

When products prepared from $dCP^{32}PP$, $dAP^{32}PP$, or $TP^{32}PP$ as the only deoxynucleoside triphosphate were hydrolyzed in this manner, each of the four 3′-deoxynucleotides of thymus DNA was found to be radioactive (experiments

1–3, Table 2). This result shows that the single deoxynucleoside triphosphates had formed 3′,5′-phosphodiester linkages (Fig. 1) to each of the four deoxynucleotides of thymus DNA.

When a product prepared from C^{14}dCPPP as the only deoxynucleoside triphosphate was hydrolyzed to 3′-deoxynucleotides, two-thirds of the recovered radioactivity appeared in deoxycytidine and one-third in 3′-deoxycytidylate (experiment 4, Table 2). Since deoxynucleosides formed in this hydrolysis could arise only from terminal deoxynucleotides of DNA (Fig. 1), two-thirds of the C^{14}dCPPP must have been located at the deoxynucleoside ends of DNA chains. The remaining one-third probably had also reacted with ends of chains but was

TABLE 2

HYDROLYSIS OF PRODUCTS TO 3′-DEOXYNUCLEOTIDES

Expt. No.*	Substrate for Preparation of Product	Products of Hydrolysis	Per Cent of Recovered Radioactivity†
1	dCP^{32}PP	3′-Deoxyadenylate	18
		3′-Deoxycytidylate	34
		3′-Deoxyguanylate	23
		3′-Thymidylate	25
2	dAP^{32}PP	3′-Deoxyadenylate	57
		3′-Deoxycytidylate	6
		3′-Deoxygunaylate	22
		3′-Thymidylate	15
3	TP^{32}PP	3′-Deoxyadenylate	17
		3′-Deoxycytidylate	10
		3′-Deoxyguanylate	21
		3′-Thymidylate	52
4	C^{14}dCPPP	Deoxycytidine	67
		3′-Deoxycytidylate	33

* In Experiments 1, 2, 3, and 4, respectively, specific activities at the time of chromatography were 5.4×10^7, 7.5×10^7, 5.4×10^6, and 1.4×10^7 c.p.m. per μmole.

In Experiment 3 the hydrolysate was desalted by adsorption on Norit and eluted with ammoniacal ethanol, and then the products of hydrolysis were separated by paper electrophoresis (Markham and Smith, *Biochem. J.*, **52**, 552, 1952). In Experiments 1, 2, and 4 the hydrolysate was chromatographed on Dowex 1 formate columns (Cunningham, Catlin, and De Garilhe, *J. Am. Chem. Soc.*, **78**, 4642, 1956; and Sinsheimer, *J. Biol. Chem.*, **208**, 445, 1954). Deoxycytidine was determined as counts not held by a Dowex 1 formate or a Dowex 1 chloride column at pH 10. This radioactivity not held by the column contained less than 0.5 per cent of the total optical density of the 3′-deoxycytidylate and therefore could not represent 3′-deoxycytidylate unadsorbed due to overloading of the column.

On the basis of the optical density at 260 mμ, the total amounts of 3′-deoxyadenylate, 3′-deoxycytidylate, 3′-deoxyguanylate, and 3′-thymidylate recovered in experiment 1 were 30.6, 22.1, 22.9, and 30.6 μmoles, respectively. This represents 96 per cent of the optical density at 260 mμ added to the column.

† The recoveries of radioactivity after chromatography or electrophoresis were 83, 93, 78, and 83 per cent in experiments 1, 2, 3, and 4, respectively.

followed by another C^{14}-deoxycytidylate to produce terminal "runs" of C^{14}-deoxycytidylate. This possibility is supported by the disproportionately high radioactivity found in 3′-P^{32}-deoxycytidylate in experiment 1 of Table 2. Also in products prepared from dAP^{32}PP or TP^{32}PP, "runs" of the incorporated deoxynucleotide are indicated similarly by experiments 2 and 3 of Table 2. It cannot be decided from these data whether these "runs" occur frequently and are very short or whether they occur rarely and are long.

Kinetics of Hydrolysis of Products to 5′-Deoxynucleotides Demonstrating the Location of Incorporated Deoxynucleotides at the Deoxynucleoside Ends of DNA Chains.—To confirm that the incorporated radioactive deoxynucleotides are located at the deoxynucleoside ends of DNA chains, a reagent was sought which would hydrolyze the products stepwise from the deoxynucleoside ends so as to

release all the radioactivity at a time when few of the total deoxynucleotides had been released. Laskowski *et al.*[14] and Boman[15] have suggested that snake venom phosphodiesterase attacks DNA stepwise from one end. Recently, Khorana *et al.*[16] have shown that this phosphodiesterase hydrolyzes synthetic thymidine oligonucleotides stepwise from the deoxynucleoside end, and with thymidine oligonucleotides bearing a 3′-acetyl group at the deoxynucleoside end the first product to appear was the 3′-acetyl derivative of 5′-thymidylate. Singer, Hilmoe, and Heppel[17] have obtained a similar result with oligoribonucleotides.

When the products were treated with venom phosphodiesterase, nearly all (>90 per cent) of the radioactivity was liberated as acid-soluble deoxynucleotides at a time when less than 3 per cent of the deoxynucleotides had been released, judged by the appearance of acid-soluble ultraviolet absorbing material (Fig. 2). Such a result was obtained for products prepared from dAP^{32}PP, TP^{32}PP, or C^{14}dCPPP. This finding provides independent evidence that the radioactivity in these products is located at or near the deoxynucleoside ends of DNA chains. It may be inferred from these kinetic studies that the phosphodiesterase does not remain attached to a DNA molecule, degrading it sequentially to completion, but that, instead, other DNA molecules compete favorably with the polydeoxynucleotide product of the enzyme action.

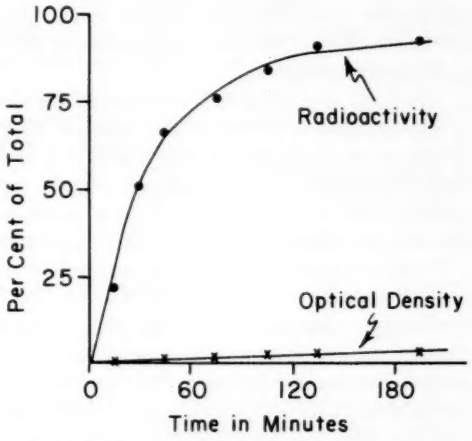

FIG. 2.—Action of venom phosphodiesterase on product. The product used in this experiment was from a reaction with C^{14}dCPPP as the only deoxynucleoside triphosphate. The reaction mixture contained 3 mg. of product, 2 units[7] of snake venom phosphodiesterase, 50 μmoles of $MgCl_2$, and 200 μmoles of tris(hydroxymethyl)aminomethane at pH 8.5 in 5.0 ml. At the times indicated, 0.5-ml. aliquots were removed, 0.25 mg. of DNA was added as carrier, and the reactions were stopped by the addition of 0.5 ml. of 1 N cold perchloric acid. Radioactivity and optical density at 260 mμ were measured in the supernatant fractions. At 200 minutes, 50 more units of diesterase were added, which resulted in total release of both radioactivity and optical density in less than 3.5 hours.

DISCUSSION

In these experiments a reaction has been observed in which a DNA molecule (containing about 20,000 deoxynucleotides) adds at its deoxynucleoside end one or a very few molecules of a single deoxynucleoside triphosphate to form a 3′,5′-phosphodiester bond (Fig. 1). Several alternative mechanisms appear to have been eliminated.

1. A non-covalent binding of the deoxynucleoside triphosphate to the DNA is excluded by the demonstrated phosphodiester linkage between the incorporated deoxynucleotide and the three other deoxynucleotides of DNA.

2. Pyrophosphorolysis of the added DNA to supply the missing three deoxynucleoside triphosphates, followed by *de novo* synthesis of DNA chains, seemed from the start an unlikely hypothesis for explaining the reaction between DNA and

a single deoxynucleoside triphosphate, because levels of inorganic pyrophosphate 100 times greater than those formed in the reaction are necessary to produce significant pyrophosphorolysis.[3] This possibility was eliminated by the finding that the incorporated deoxynucleotide is located exclusively at or near the ends of DNA chains.

3. *De novo* formation of chains containing exclusively the single deoxynucleotide is eliminated by the demonstrated phosphodiester linkage between the single deoxynucleoside triphosphate and each of the three other deoxynucleotides of thymus DNA.

4. Reaction of the single P^{32}-deoxynucleoside triphosphate with the non-nucleoside end of the DNA chain[18] would not have resulted in the appearance of P^{32} in the three other deoxynucleotides of DNA when the product was hydrolyzed to 3′-deoxynucleotides.

The basis of a sensitive tool for analyzing the number and kinds of end-groups of a homogeneous DNA could be furnished by reacting such a DNA sample with a single deoxynucleoside triphosphate and then hydrolyzing the product to 3′-deoxynucleotides. Since nucleases which contaminate the purified enzyme could produce new ends or remove deoxynucleotides added in the reaction, extension of these studies in this direction must await further purification of the enzyme.

What is the pertinence of this reaction between DNA and a single deoxynucleoside triphosphate to the extensive DNA synthesis observed when all four deoxynucleoside triphosphates are present? It appears likely that it is the same highly purified *E. coli* enzyme which is responsible for both reactions. One possibility is that the reaction between DNA and a single deoxynucleoside triphosphate may actually be the first step in the over-all process of enzymatic replication of the DNA primer; then the terminal replicating chain, formed when four deoxynucleoside triphosphates are present, would loop back, in order to orient itself along the primer chain. It is conceivable that such loops could be produced by the rotation of a very few deoxynucleotide units and could be dissipated at a later time by nuclease action. Another possibility is that the reaction between DNA and a single deoxynucleoside triphosphate is merely part of the extension of the shorter of two unequally long chains of a DNA double helix and is not directly related to the replication of intact DNA molecules. In this event replication of DNA would not involve a covalent binding between the primer and the replicating chain; instead, the deoxynucleoside triphosphates would first form hydrogen bonds to corresponding deoxynucleotides of the primer chain and would then polymerize. It is evident that more must be learned about the chemistry of the DNA primer itself as well as about these enzymatic reactions before these questions of mechanism can be answered.

SUMMARY

1. The reaction between deoxyribonucleic acid (DNA) and a single radioactive deoxynucleoside triphosphate resulted in a product whose radioactivity sediments at the same rate as the average DNA molecules; this finding indicates that the deoxynucleotide had become incorporated into molecules which have the same size as DNA.

2. Hydrolysis of the product to 3′-deoxynucleotides demonstrated the formation

of 3′,5′-phosphodiester linkages between the deoxynucleoside end of a DNA chain and the added deoxynucleotide. These studies further showed that only one or a very few molecules of the single deoxynucleoside triphosphate reacted with the end of each chain.

3. The kinetics of hydrolysis of the product by snake venom phosphodiesterase, involving a sequential liberation of deoxynucleotides from the deoxynucleoside end of DNA chains, provided independent evidence that the deoxynucleoside triphosphate reacted with the deoxynucleoside end of a DNA chain.

This investigation was supported by research grants from the National Institutes of Health, Public Health Service, and the National Science Foundation. We are grateful to Dr. Eloise E. Clark for performing the ultracentrifugal analyses and to Dr. Howard K. Schachman for valuable advice and discussions.

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[1] Abbreviations used: DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; dAPPP, deoxyadenosine triphosphate; dCPPP, deoxycytidine triphosphate; dGPPP, deoxyguanosine triphosphate; TPPP, thymidine triphosphate.

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VITAMIN A DEFICIENCY AND NIGHT BLINDNESS

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Communicated May 16, 1958

One of the oldest diseases known to man is nutritional night blindness. Its descriptions go back to the ancient Egyptian medical papyri and are already accompanied by the correct prescription for its cure, the eating of liver. Toward the end of World War I the factor in liver which cures night blindness was identified with the then newly discovered vitamin A.[1]

Vitamin A is the precursor in the retina of the visual pigments of the rods and cones.[2] It seems reasonable to suppose that on a diet deficient in this factor the retina eventually synthesizes subnormal amounts of visual pigment, with the corresponding decline of visual sensitivity that constitutes night blindness.

Some of the first studies of experimental human night blindness seemed to reveal such a simple and direct relationship.[3] In two subjects deprived of vitamin A, the visual thresholds of both rods and cones began at once to rise, until a mild night blindness had been established.[4] On oral administration of vitamin A or carotene, the thresholds of both rod and cone vision returned to normal within 2–3 hours.

It looked for a time, therefore, as though this might be an exemplary instance of the origin and cure of a biochemical disease, all elements of which were well understood. Further studies, however, exposed two major discrepancies: (1) Though in some subjects placed on a vitamin A–deficient diet the visual threshold began at once to rise, in a larger number it remained unchanged for periods ranging from several months[5] to, in one instance, 2 years.[6] (2) Among the subjects who developed night blindness, some were completely cured within a few hours after receiving vitamin A, whereas others, though showing some immediate improvement, took months of vitamin A supplementation to return to normal.[7]

One might take a simple position with regard to the first of these discrepancies. The amounts of vitamin A stored in the livers of healthy human subjects are known to vary enormously.[8] In Britain, for example, Moore[6] found reserves in adults during 1941–44 ranging from about 7 to 750 μg/gm. If we take 1,500 gm. as the average weight of the adult liver and about 300 μg. (about 1,000 I.U.) as the daily drain upon stored vitamin A, the average Briton stores enough vitamin A in his liver—if used economically—to tide him over some 500 days of total deprival. An unusually well-supplied Briton—if we can disregard spoilage—might survive seven times as long, or almost 10 years! On the other hand, the most poorly supplied members of this group might have run through their stored vitamin A within 1–2 weeks. It is not difficult to understand, therefore, why most subjects taken from ordinary American or British environments fail to respond to vitamin A–deficient diets within months or even years. It is less clear why a fairly large proportion of them responded within a few days, even though in some instances highly supplemented with vitamin A for the preceding period.[3]

The second discrepancy—the great variability in the times required to cure night blindness—raises other issues. The visual pigments are composed of vitamin A

aldehyde (retinene) joined to specific proteins of the rods and cones called "opsins." The amounts of visual pigment that can be formed in the normal retina are limited, not by vitamin A, which is ordinarily present in excess, but by opsin. In thinking about night blindness, we have tended in the past to be too much preoccupied with vitamin A and have paid too little attention to the opsins.[2]

When one does consider the opsins, this at once suggests further relationships. The outer segment of a rod—and this must be true also of many cones—is composed in considerable part of visual pigment, that is, of opsin, since the retinene chromophore constitutes only about 1 per cent of these molecules. Opsin accounts for about 40 per cent of the dry weight of the outer segment of a frog rod and 14 per cent of that of a cattle rod.[2] It is an important structural constituent of the rods and probably of the cones; and any loss of this protein might be equivalent to the structural deterioration of the visual receptors.

Tansley[9] showed some years ago that in vitamin A–deficient rats and dogs, somewhat later than the decline in rhodopsin production that should have initiated night blindness, the outer segments of the rods deteriorated structurally. Johnson[10] confirmed and extended these observations in the rat; and recently similar changes have been observed in both rods and cones of the monkey.[11] According to Johnson, after 7–13 weeks of vitamin A deprivation in young rats, many outer segments have disappeared, and those that remain stain abnormally. As the deficiency progresses, the inner segments of the rods also degenerate, and then successively the external limiting membrane, the outer nuclear layer, and the inner nuclear layer. These changes occur sooner in central than in peripheral areas of the retina. The outer segments of rods which have deteriorated only slightly seem to repair considerably within 24 hours of feeding vitamin A. Even rods which have degenerated completely seem to be replaced within 10–18 weeks of vitamin A supplementation.

These observations suggest that the time required to cure night blindness may depend on the extent to which vitamin A deficiency has altered the retinal structure. Simple lack of vitamin A, through lowering the concentrations of visual pigments, might induce a night blindness that is cured as rapidly as vitamin A re-enters the retina; but the structural deterioration of the retina, heralded perhaps by the loss of opsin, might take much longer to repair.

For these reasons it seemed worthwhile to map the entire course of vitamin A deficiency and its cure in the rat. In single groups of animals we have measured simultaneously the vitamin A in the liver and blood, the retinal content of rhodopsin and opsin, the electroretinographic threshold, and the ERG's obtained over a wide range of light intensities. In key instances we have also examined the retinal histology.

Not all these things were done for the first time. We have already discussed the histological studies of Tansley and Johnson and should mention particularly also Tansley's fine study of rhodopsin synthesis in normal and vitamin A–deficient rats[12] and the measurements of liver, blood, and retinal vitamin A in normal and deficient rats by Lewis, Bodansky, Falk, and McGuire.[13]

Plan of the Experiments.—A number of experiments were performed, all of which yielded substantially the same pattern of results. We shall describe primarily the last such experiment, because it brings together all the procedures and represents most completely and typically all our observations.

Male albino rats of the highly inbred Harvard colony, 22–24 days old and weighing 36-66 gm., were divided into two groups, one kept on the complete laboratory ration, the other placed on the standard USP vitamin A test diet. The animals on the deficient diet continued to gain weight for about 5 weeks, though more slowly than normal. At this time they weighed an average of 112 gm. as compared with the control weight of 215 gm. but were altogether normal in appearance. In the fifth to seventh weeks their weights plateaued and thereafter declined rapidly. At the same time—in the seventh and eighth weeks— the classic overt signs of vitamin A deficiency appeared, and by the end of the eighth week all the animals not sacrificed in the experiments had died.

For electroretinography, animals that had been dark-adapted overnight were anesthetized with nembutal. The eye was held open with threads drawn through the lids. Cotton-wick electrodes were used, moistened with Ringer solution, one touching the side of the cornea, the other a shaved area on the cheek. The response was recorded with a capacity-coupled Grass P4 preamplifier and a Dumont oscilloscope with camera attachment. The stimuli were $^1/_{50}$-second flashes of white light, the intensity of which was controlled with neutral filters and photographic wedges. The absolute threshold was measured by starting with the light well below threshold and flashing it every few seconds at gradually increasing intensities until a response could be detected on the oscilloscope. This procedure was repeated until constant readings were obtained. Then the ERG was recorded over a wide range of intensities.

After dark-adapting overnight, the same animals were used next morning for the biochemical measurements. They were again anesthetized, the body cavity was opened, and 5–10 ml. of blood were taken from the heart with an oxalated syringe. The entire liver was removed and also both eyes. One eye of each animal was used to measure rhodopsin, the other to measure opsin; but, since each of these determinations requires 2 retinas, animals were paired, usually on the basis of having yielded comparable electroretinograms.

To determine blood vitamin A, the oxalated blood from one animal was centrifuged, and the clear plasma was mixed with an equal volume of ethyl alcohol and extracted three times with petroleum ether. This extract was transferred to 0.3 ml. of chloroform, and a micro-antimony chloride test was performed by mixing 0.25 ml. of the extract with 0.50 ml. of antimony chloride reagent, recording the absorption spectrum at once in a Cary recording spectrophotometer.

The livers, weighing 4–12 gm., were ground with anhydrous sodium sulfate to a fine powder and extracted by shaking with diethyl ether. An aliquot of this extract was transferred to chloroform, and its vitamin A content determined by the antimony chloride procedure.

To measure rhodopsin, two retinas were hardened in 4 per cent alum solution for 15–20 minutes, then washed with distilled water and buffer, and extracted overnight with 0.2 ml. of 2 per cent digitonin solution. After centrifuging, 0.01 ml. of 1 *M* hydroxylamine was added to the extract, and the absorption spectra recorded before and after bleaching. The change in extinction at 500 mμ measured rhodopsin.

Opsin was determined by measuring the capacity of retinas to regenerate rhodopsin when incubated with neo-*b* (11-*cis*) retinene.[14] We found that rat rhodopsin

in digitonin solution regenerates very little when bleached and incubated with neo-*b* retinene. For this reason whole retinas were exposed to bright light until wholly bleached. Then a large excess of neo-*b* retinene, dissolved in 0.025 ml. acetone, was added to the retinas suspended in buffer solution, the mixture was stirred periodically during 4–6 hours at room temperature and then left at 5° C. overnight. The rhodopsin which had formed was extracted and measured as described above. Control measurements showed that 70–80 per cent of rhodopsin originally present in a retina was regenerated and recovered by this procedure.

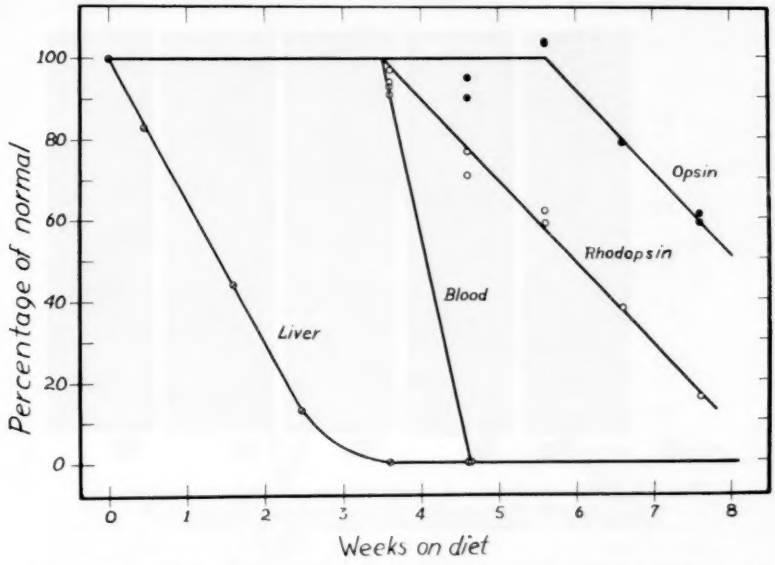

FIG. 1.—Biochemical changes in a group of white rats on a vitamin A–deficient diet. The animals were 22–24 days old when the diet was begun. The liver vitamin A began to fall at once and within 3 weeks had reached low values. Then within a week the blood level fell from normal to zero. With this, the rhodopsin content of the retina declined, marking the onset of night blindness. Later the opsin also declined, marking the beginning of the histological deterioration of the retina.

Vitamin A of Liver and Blood; Rhodopsin and Opsin.—Figure 1 shows in one group of animals the effects of the deficient diet on the vitamin A content of the liver, the vitamin A concentration in the blood, and the rhodopsin and opsin of the retina. The values are expressed as percentages of normal. For the liver this means the percentage remaining of the vitamin A present in control animals at the time the diet was begun. The blood vitamin A, rhodopsin, and opsin are expressed as percentages of the values found in control animals of the same age.

The liver vitamin A begins to fall as soon as the diet is begun and within 3 weeks has reached a very low value. This depletion proceeded at the average rate of 2–2.5 μg. daily, the withdrawal rate for animals of this age and weight. Meanwhile, the control animals on the complete diet increased their liver stores at an average rate of 45 μg. daily. It is this that makes the age at which the diet is

begun decisive for the course of the deficiency. Our control animals when 53 days old had livers weighing, on the average, 16 gm. and containing 1,360 μg. vitamin A; withdrawn at a daily rate of even 5 μg., this might have tided them over 9 months of a deficient diet.

The blood maintains its normal concentration of vitamin A (10.4 μg. per cent in the deprived animals, 11.2 μg. per cent in the controls) until the liver has been

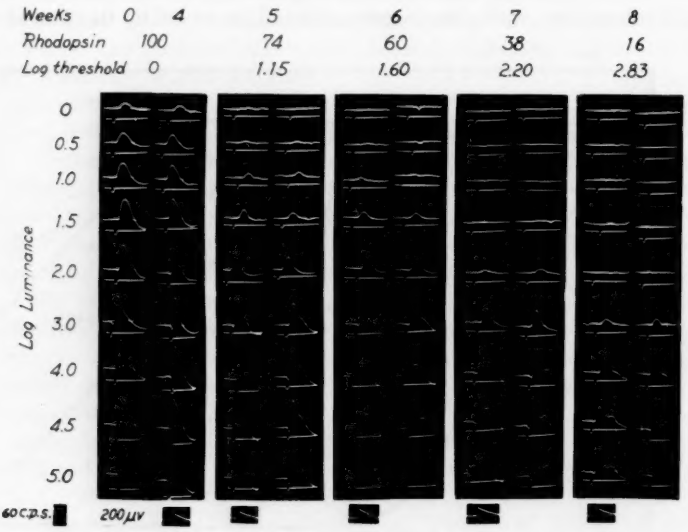

FIG. 2.—Effects of vitamin A deficiency on the electroretinogram (ERG). The top three lines show the number of weeks on the deficient diet, the rhodopsin content of the retinas as percentages of normal; and the logarithm of the luminance of light needed to evoke a just perceptible ERG, the average threshold of normal animals being set arbitrarily at 1 (log threshold = 0). ERG's are shown in response to a range of luminances of 5 log units, i.e., 1 to 100,000. Below each ERG, a marker shows the $^1/_{50}$-second flash. The small rectangles at the bottom show a trace of the 60-cycle A.C. to indicate the time scale, and weekly calibrations of the oscillographic response to a pulse of 200 microvolts. The first two vertical rows of ERG's show responses from a rat about to begin the diet and those from another after 4 weeks, when the vision is still normal. Thereafter, records are shown from a pair of animals each week. As the rhodopsin declines, the ERG threshold rises (night blindness), and the ERG displays characteristic changes: (*a*) the *b*-wave at each level of luminance declines; (*b*) the *a*-wave declines still more rapidly; and (*c*) an inflection on the downward sweep of the *b*-wave is delayed longer and longer until it appears as a separate positive wave.

emptied. Then in the space of a few days the blood vitamin A falls precipitately to zero.

Up to this time the rhodopsin content of the retina remains normal. The extract of two retinas in 0.21-ml. solution possesses an extinction at 500 mμ of 0.280, corresponding to a rhodopsin content of 7.24×10^{-4} μmoles per retina. This is equivalent to a vitamin A content of 0.21 μg. per retina. (Lewis *et al.*[13] found only one-fifth to one-third as much vitamin A in the rat retina; the description of their preparative procedure suggests that it may have involved large losses of rod outer segments.)

Now the rhodopsin also begins to fall and within 3 weeks has reached very low values. As we shall see, this marks the beginning of night blindness.

There is a curious interval of 2–3 weeks in which, though the rhodopsin content has declined, the opsin level is still normal. That is, with the liver and blood emptied of vitamin A, the retina contains opsin which can find no vitamin A with which to combine.

Then the opsin level, too, begins to fall, and this marks the beginning of the structural deterioration of the retina (see below). At this time also—the seventh and eight weeks of the diet—the classic signs of vitamin A deficiency appear. (In another of our experiments it took 10–11 weeks to reach this stage.) The

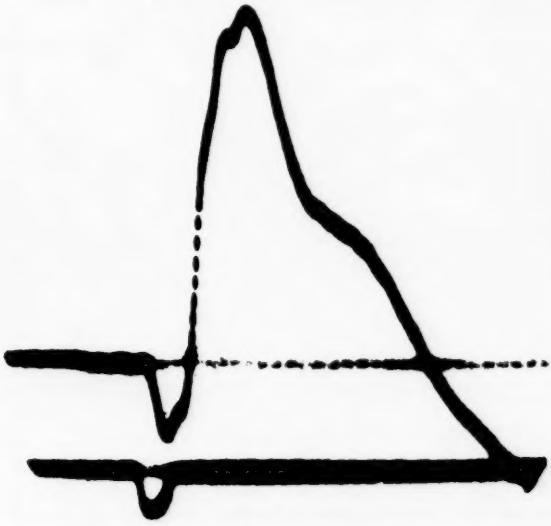

FIG. 3.—Form of the ERG. Both the *a*- and *b*-waves are two-cusped. This feature appears also in the human ERG, where the shorter-latency component in each wave is believed due to cone responses, the longer-latency component to rods. The strong inflection evident on the downward sweep of the *b*-wave is delayed longer and longer as night blindness develops. Below the ERG, a marker shows the $^1/_{50}$-second stimulating flash.

animals lose weight rapidly; and by the end of the eighth week all not already used in the experiments have died.[15]

Physiological Changes.—For the first 4 weeks on the diet, the animals appear to be physiologically normal. During this time, first the liver and then the blood is depleted of vitamin A. In the fifth week, as the rhodopsin level begins to fall, the visual threshold rises, marking the beginning of night blindness.

Figure 2 shows electroretinograms recorded in pairs of experimental animals. At the top of the figure are shown the number of weeks on the diet, and below this the average rhodopsin content of the retinas as percentages of the normal value. Below this is the logarithm of the threshold for a just perceptible retinogram; the normal threshold has been set arbitrarily at 1 (log threshold = 0), so that these

numbers represent the rise in log threshold over the normal value. The rat's eyes were exposed to $^1/_{50}$-second flashes at the log luminances shown on an arbitrary scale at the left. The figure shows a series of ERG's at various luminances for each of two rats each week. Since no changes occur in the first 4 weeks, the first pair of records involves one rat just about to begin the diet and another rat that had been 4 weeks on the diet.

At the end of the fourth week the ERG is entirely normal. In the fifth week, the rhodopsin level falls to 74 per cent, and the visual threshold rises 1.15 log unit, or about 14 times. In the succeeding weeks, as the rhodopsin level continues to

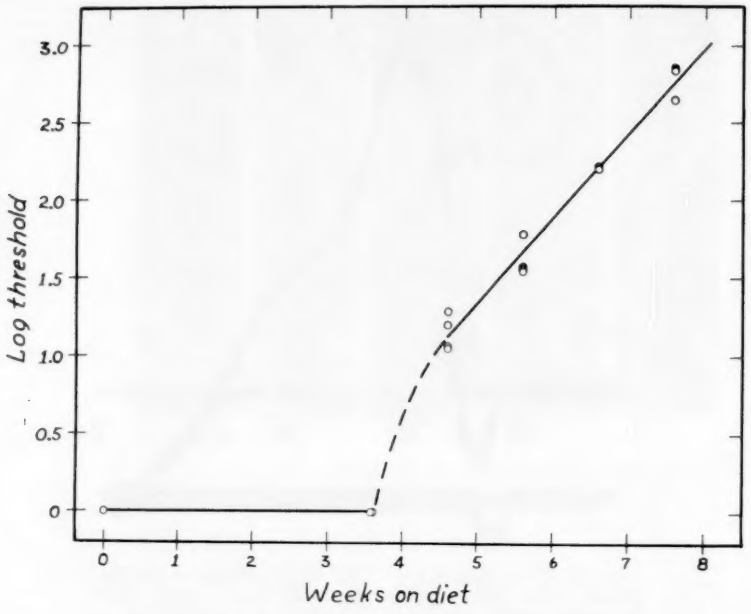

FIG. 4.—Development of night blindness with time on the vitamin A–deficient diet. Each point shows the logarithm of the ERG threshold of a single animal, the normal threshold being set arbitrarily at 1 (log threshold = 0). The log threshold rises linearly with time on the diet, following a more rapid initial rise.

decline, the threshold rises until, at the end of the eighth week, the rhodopsin is at 16 per cent, and the visual threshold has risen about 680 times.

Simultaneously, the ERG undergoes characteristic changes: (1) At all luminances but particularly at the lower ones, the height of the positive *b*-wave declines as the deficiency progresses. So, for example, at log luminance 3.0 the *b*-wave, nearly maximal at the end of the fourth week, has sunk to the just perceptible threshold level by the end of the eighth week. (2) The negative *a*-wave declines in amplitude still more rapidly. It is initially nearly as large as the *b*-wave at log luminance 5; but by the end of the eighth week it can hardly be elicited at all, even at this highest luminance. (3) A small positive inflection, which appears initially only as a hump on the downward sweep of the *b*-wave, is delayed longer and longer as the

deficiency progresses, until finally it has become a well-separated second positive wave, particularly evident at the higher luminances. The source of this delayed positive wave has not yet been identified. The possibility that it is an off-effect is not supported by our tests.

It may be noted in passing that some of the ERG's show clearly that both the *a*- and the *b*-waves are two-cusped. This is particularly evident in such records as those of the sixth week at log luminance 5. One of this pair of ERG's has been enlarged in Figure 3. Such two-cusped *a*- and *b*-waves have come to be associated in the human retinogram with the responses of cones and rods, the shorter latency component in each wave presumably representing the cone response.[16]

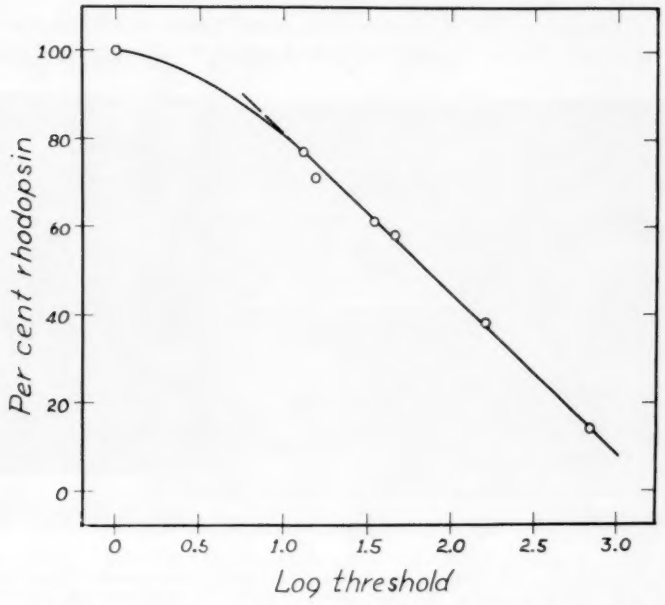

FIG. 5.—Rise of the ERG threshold with decline in rhodopsin content in the retinas of vitamin A–deficient rats. The normal log threshold is set at 0. Rhodopsin is expressed as the percentage of that found in control animals of the same age. Over most of its course the log threshold rises linearly as rhodopsin falls, following a disproportionately large rise of threshold with the first decline of rhodopsin.

It has frequently been asserted that rats possess only rods; yet Tansley[15] and Walls[17] state unequivocally that cones are present, and Sidman[18] has recently reported finding them in the approximate proportion 1 cone:10 rods. Since in the whole human retina the proportion of cones to rods is about 1:20, there may be as good anatomical basis in the rat as in man, for the two-cusped ERG's to represent cone and rod responses.

Figure 4 shows the relation between log threshold, and time on the deficient diet. After a somewhat abrupt start in the fifth week, the log threshold rises linearly. Similar behavior has been reported in man, the log threshold, rod and cone, rising linearly or nearly linearly for long periods on vitamin A–deficient diets.[3, 7]

Figure 5 shows the relation between the rhodopsin level of the retina and the logarithm of the visual threshold. Most of this relation again is linear, though distorted, as was Figure 4, by a somewhat disproportionate rise of threshold accompanying the first decline of rhodopsin. When the rhodopsin has fallen to half its normal content as the result of the deficiency, the threshold has risen about 100 times; when the rhodopsin is at 10 per cent, the threshold is up about 1,000 times. It would be interesting to determine whether in normal rats the bleaching of rhodopsin that accompanies light adaptation causes similar changes of threshold. It is now recognized that in the human eye the bleaching of a small fraction of rhodopsin raises the threshold enormously;[19] by one estimate, the bleaching of 0.6 per cent of the rhodopsin raises the threshold about 3,300 times.[20]

Histologic Changes.—The deterioration of retinal tissues in vitamin A–deficient rats has been described by Tansley[9] and by Johnson.[10] Our principal problem is

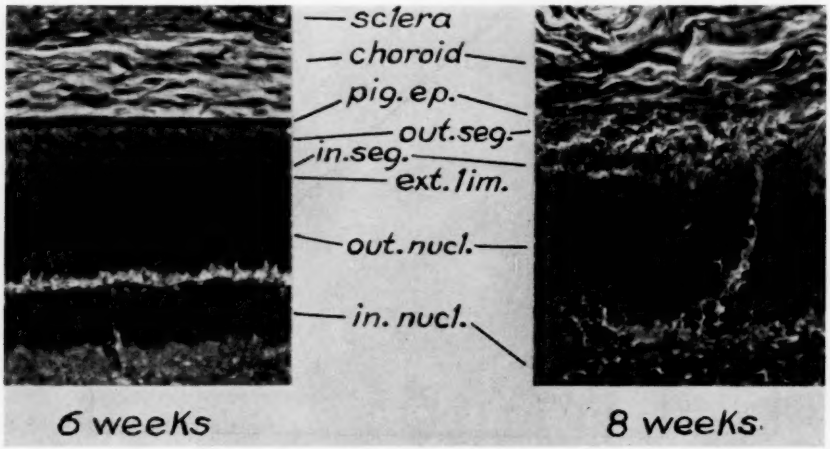

FIG. 6.—Effect of vitamin A deficiency on the structure of the rat retina. After 6 weeks on a vitamin A–deficient diet, the retina appears entirely normal. After 8 weeks it has deteriorated markedly: only traces remain of the pigment epithelium; the layer of rods, particularly the outer segments, is disintegrating; and the external limiting membrane has disappeared.

to orient these changes in the pattern of biochemical and physiological events that we have described.

After 6 weeks on the vitamin A–deficient diet, when the rhodopsin had fallen to about half its normal value but the opsin was still intact, the retinal histology of these animals appeared entirely normal (Fig. 6, *left*). After 8 weeks on the diet, however, when the opsin also had fallen to about half its normal value, the retinal tissues had deteriorated markedly (Fig. 6, *right*). The outer segments of the rods were attenuated, many had a gnawed appearance, and they were irregularly spaced. Only vestiges of the pigment epithelium remained. The sharp boundary that marked the external limiting membrane was gone; and the blood vessels of the choroid layer were frequently occluded.

Our supposition that, when opsin goes, the outer segments of the rods should deteriorate structurally has proved to be correct. By this time, however, the

animal is deteriorating generally. Not only are other retinal tissues affected as just described, but the superficial structures of the eye now begin to display the classic signs of vitamin A deficiency: corneal clouding, xerophthalmia, and secretion of a sticky red exudate about the eyes. The animal is losing weight rapidly, the coat is disarranged, some animals have developed an unsteady gait, some breathe with difficulty.

There is no compelling reason, therefore, to single out opsin among the animals' disabilities. To the degree that the loss of opsin is responsible for the histological decay of the outer segments of the rods, it may be only one of many proteins respon-

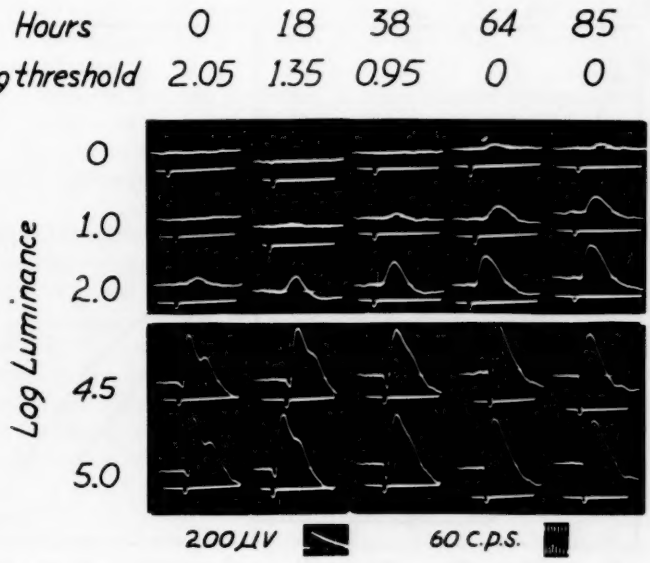

FIG. 7.—Recovery from night blindness on administration of vitamin A. Following intraperitoneal injection of a large dose of vitamin A, the ERG threshold returns within 64 hours to normal (log threshold = 0). During this interval the ERG retraces in reverse all the changes which had accompanied the development of night blindness.

sible for similar manifestations in many tissues. We shall have more to say of this below.

Recovery from Night Blindness.—A number of animals which have developed night blindness were "cured" by administering vitamin A. It was found that the vitamin could be supplied more effectively by intraperitoneal injection than orally. Oral administration yielded irregular results, successful in some deficient animals, not in others. It seemed as though some deficient animals had lost temporarily the capacity to take up vitamin A fed by mouth.

The effect of administering vitamin A to a night-blind animal is shown in Figure 7. At the beginning of the experiment this animal exhibited much the same ERG responses as did the animals of Figure 2 in the seventh week of the deficiency. Its ERG threshold was a little more than 100 times normal.

This animal was given a large dose of vitamin A—340 μg. dissolved in 1 ml. cottonseed oil, injected intraperitoneally. The ERG threshold slowly fell and within 64 hours had reached the normal level. Simultaneously, the ERG retraced in reverse all the changes that had accompanied the development of night blindness: the *a*- and *b*-waves increased to their former sizes, and the delayed positive wave was reincorporated into the *b*-wave.

Figure 8 shows the return of the ERG threshold to normal in a series of such recovery experiments. On the left are shown data from three deficient animals exhibiting various degrees of night blindness. Various amounts of vitamin A in cottonseed oil, ranging from 320 to 920 μg., were injected in 1–3 doses. In every

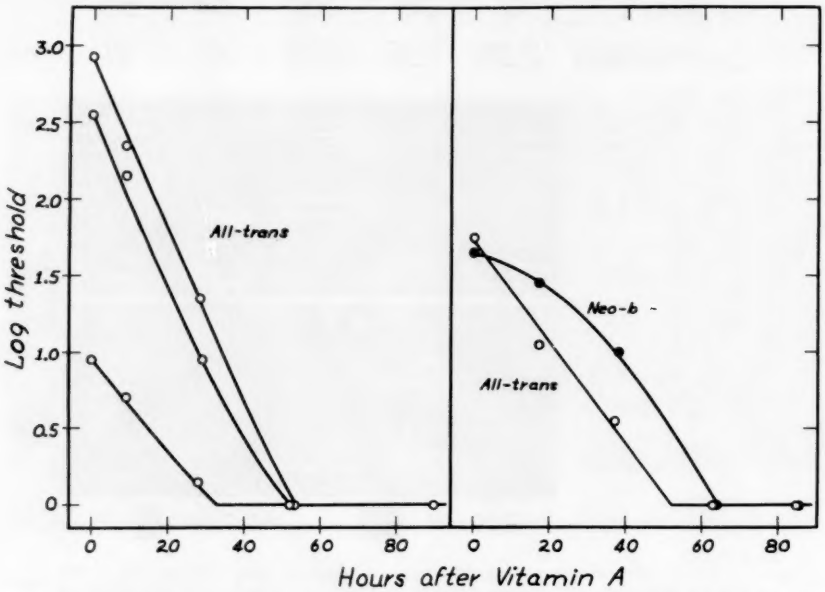

FIG. 8.—Recoveries from night blindness on administration of vitamin A. Log ERG thresholds measured at various times after intraperitoneal injection of large doses of all-*trans* vitamin A (*open circles*), or neo-*b* (11-*cis*) vitamin A (*closed circles*). After injection of the all-*trans* isomer, the log threshold falls almost linearly to the normal level; but with neo-*b* vitamin A there is a lag in recovery, caused apparently by the necessity to isomerize this configuration primarily to all-*trans* before it is used by the eye.

case the fall of log threshold to the normal level was approximately linear. The time for complete recovery depended primarily on the degree of night blindness, not the dosage level, at least within this range of high dosage.

In one instance two rats, closely matched in their degree of night blindness, were injected with equal amounts (*ca.* 1,000 μg.) of two geometric isomers of vitamin A: the all-*trans* form, which is most prevalent and which has been shown to be most effective in stimulating growth and liver storage in the rat,[21] and the hindered *cis* neo-*b* isomer (11-*cis*), which serves as precursor of the visual pigments. The result is shown at the right in Figure 8. The fall of log threshold on injection of all-*trans* vitamin A was, as usual, approximately linear; the neo-*b* isomer, however,

exhibited a distinct initial lag in its effect, though after 30–40 hours the responses to both isomers ran approximately parallel.

The vitamin A of the liver and blood was measured in these animals and in another pair which had been treated similarly. The animals injected with all-*trans* vitamin A had stored 71–87 μg. in the liver, in each case about 9 per cent of that injected, whereas those that received neo-*b* vitamin A had stored 32–39 μg., or about 4 per cent of that injected. Isomerization experiments conducted in both instances showed that none of the stored vitamin A was of the neo-*b* configuration. The blood levels after injection of either all-*trans* or neo-*b* vitamin A were normal (11.9 and 13.8 μg. per cent, respectively); again none of this was neo-*b*. Rhodopsin also was extracted from the retinas of the animals which yielded the data of Figure 8 (*right*). The extinctions at 500 mμ were 0.259 and 0.252, somewhat low, yet within the normal range.

It appears, therefore, that neo-*b* vitamin A, when injected intraperitoneally, is converted to other isomers, primarily all-*trans*, before entering the eye. It is presumably this process of isomerization that causes the observed delay in its action and may be responsible also for its relatively low effectiveness in growth and storage.[21]

Where this isomerization occurs is not yet known; but it appears to be a general feature of vitamin A metabolism, for surveys in our laboratory of representative tissues in fishes, rats, and cattle have failed to find the neo-*b* isomer anywhere but in the eye.[22] It seems likely that only other isomers of vitamin A are carried in the blood or stored in the liver and that the eye tissues themselves isomerize one or several of these to the neo-*b* configuration for the synthesis of visual pigments. The bleaching of the visual pigments by light, on the other hand, yields all-*trans* retinene and vitamin A; so that the visual processes include a continuous cycle of geometric isomerization between these two configurations.[14]

At the left of Figure 8 two recoveries are shown from states of night blindness involving 2.5–3 log units rise of threshold above normal. Figure 5 shows that this degree of night blindness corresponds to the decline of rhodopsin to 10–20 per cent of normal and Figure 1 that, by this time, opsin should have fallen to about 60 per cent of normal, with a corresponding disturbance of retinal histology. We cannot be certain that all these lesions occurred in the animals that yielded the data of Figure 8, but they were to be expected. The return of the threshold of these animals to normal 50 hours after administration of vitamin A implies that, within this interval, not only had neo-*b* vitamin A again been made available but that the retina had regained whatever opsin it had lost and had repaired whatever tissue deterioration had occurred. It should be said at once that these animals had been selected because their corneas were clear, since we wished to avoid the special complications of threshold measurement with cloudy corneas. The animals were, however, losing weight, and some of them exhibited the rough coats, red eye exudate, and postural imbalance already noted. In such instances, though a single large dose of vitamin A quickly brought the visual threshold back to normal, as shown in Figure 8, the external appearance was still poor. Several days elapsed before the weight began to increase, and it took several weeks before they again looked sleek, though no more vitamin A had been administered.

Conclusions.—These experiments reveal a simple and consistent series of changes

directly related to the availability of vitamin A. On being deprived of vitamin A, the rat exhausts its store of this substance in the liver at a regular rate, meanwhile maintaining the levels in the other tissues. With the liver emptied, the blood level falls, and shortly afterward evidences of tissue deprivation appear. The first of these is night blindness—the visual pigments lose their prosthetic group, vitamin A aldehyde. Later the protein components of the visual pigments, the opsins, also decline; but this is a secondary phenomenon accompanied by general signs of tissue disintegration in the eye and elsewhere in the organism. On administering vitamin A, all these changes are reversed.

For some years past we have had to face the embarrassment that the only function of vitamin A in the organism that we understand—that of supplying the chromophores of the visual pigments—plays only a trivial part in the whole complex of vitamin A deficiency. No animal dies of night blindness. Vitamin A must have some general and fundamental function in tissues all over the body, perhaps particularly in epithelia, so that in its absence the organism as a whole deteriorates and eventually succumbs. The nature of this general function is still unknown.

It has recently become increasingly evident that the protein opsin is stabilized chemically by its combination with retinene. Opsin, for example, is denatured much more readily than rhodopsin, by acids and alkalies[23] and by heat.[24] We have supposed that possibly for this reason opsin can be maintained in the rods and cones only through its opportunity to form visual pigments; and that, when this opportunity is lost, as in vitamin A deficiency, the opsins also might soon be lost. It is in part this consideration that led us to perform the present experiments.

They come out well in this regard. It is true, whether for this reason or another, that, shortly after opsin ceases to be saturated with retinene, it begins to leave the retina; and, since opsin is an important structural constituent of the outer segments of the rods, it is perhaps for this reason that the rods deteriorate anatomically. The important point is that these things do not happen alone but as part of a wide pattern of tissue disintegration all over the body. The outer segments of the rods are only one among many tissues, and opsin may be only one of many tissue proteins stabilized by the presence of vitamin A or its derivatives and doomed in its absence.

We should like, therefore, to suggest that opsin may behave here as a representative protein and that the mechanism of its stabilization in the rods and cones may be representative also. This would mean that many tissues contain structural proteins which are stabilized by direct combination with vitamin A or its derivatives and which are lost with the consequent anatomical disintegration of the tissues in the absence of vitamin A. In this sense opsin and the rod outer segments may provide a model for the general function of vitamin A in the tissues.

* This investigation was supported in part by funds from the Rockefeller Foundation and the Office of Naval Research. J. E. D. wishes to express his appreciation of a summer stipend provided by the National Science Foundation. We should also like to thank Dr. Toichiro Kuwabara of the Howe Laboratory of Ophthalmology and Dr. David G. Cogan, its director, for preparing the histologic sections used in this investigation.

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to correspond to a permanent congenital failure of rod function. We know of no adequate histologic studies in man. In certain mutant mice the layer of rods is missing, though the remainder of the retina appears normal (C. E. Keeler, *J. Exptl. Zool.*, **46**, 355, 1927). The present observations suggest that some forms of hereditary night blindness may possibly involve the failure to synthesize the specific protein of the rods—rod opsin; the comparable hereditary disease, day blindness, might similarly be caused by the failure to synthesize cone opsin.

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*MOLECULAR INTERACTION OF ISOALLOXAZINE DERIVATIVES**

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Communicated by Hans T. Clarke, May 7, 1958

Theorell and Kuhn and their co-workers[1–5] have presented considerable evidence in support of the view that the phosphate group of flavin mononucleotide (FMN) plays an important role in the binding of FMN to the protein component of the "old yellow enzyme," and it seems likely that a similar situation obtains in other FMN-protein systems. It has been suggested that, in the "old yellow enzyme" at physiological values of pH, the negatively charged phosphate group is bound to positively charged amino groups of the protein.[4, 5]

Less clear is the nature of the interaction between protein and the *iso*alloxazine portion of FMN. That such interaction occurs is evident from the fact that free FMN and protein-bound FMN differ in absorption spectrum, fluorescence spectrum, and oxidation-reduction potential. Moreover, addition of riboflavin to the protein component of the "old yellow enzyme" leads both to the development of enzymic activity and to quenching of the fluorescence of riboflavin.[3, 4] The results of the early investigations by Kuhn and his colleagues (reviewed extensively[6–9]), in which riboflavin analogues and derivatives were examined both for vitamin activity and for ability to give rise to enzymic activity when added to the protein of the "old yellow enzyme," the extensive antimetabolite studies performed in many laboratories,[6–9] and the recent kinetic data of Theorell and Nygaard[4, 5] are all consistent with the idea that the *iso*alloxazine part of FMN plays a key role in protein-FMN interaction.

Such interaction may be expected to differ not only between one FMN-protein and another, but also between reversibly interconvertible states and compounds of a single flavoprotein. This may hold to an even greater extent for the more complex flavin adenine dinucleotide-proteins and metalloflavoproteins. It would seem of interest to examine the linked functions[10] of flavoprotein systems in a manner analogous to the systematic study of the heme protein, horseradish peroxidase, now in progress in this laboratory.[11] However, whereas studies of heme proteins are facilitated by the availability of considerable information on the interaction of metalloporphyrins with nonprotein ligands,[12] little analogous information exists for *iso*alloxazine derivatives. In the present communication we report on studies undertaken for purposes of orientation preparatory to more detailed investigations.

EXPERIMENTAL

Materials.—3-Methylriboflavin was synthesized by condensation[13] of 2-amino-4,5-dimethyl-*N*-(1′-D-ribityl)-aniline[14] and methylalloxan.[15] Melting point: 269°–270° C., with decomposition. Analysis: calculated, C 55.37, H 5.69, N 14.36; found, C 55.43, H 5.95, N 14.37. Absorption and fluorescence spectra are shown in Figure 1.

p-Hydroxycinnamic acid (m.p. 212° C.), *p*-methoxycinnamic acid (m.p. 185° C., sinters 170° C.), and L-tyrosine ethyl ester (m.p. 166° C.) were gifts from Dr. Christine Zioudrou and Professor Joseph S. Fruton. All other compounds used were

commercial preparations, purified as required. Solutions were freshly prepared immediately prior to use. Care was taken to protect flavin-containing solutions from light.

Apparatus.—Absorption spectra were determined with the use of Cary model 11, Spectracord model 3000, and Beckman model DU spectrophotometers. Fluorescence spectra were determined with a spectrofluorometer constructed from two Bausch and Lomb grating monochromators, a Xenon arc light source, a 1P28 photomultiplier tube with battery power supply (Farrand Optical Co.), and a Varian Associates model G10 recorder.

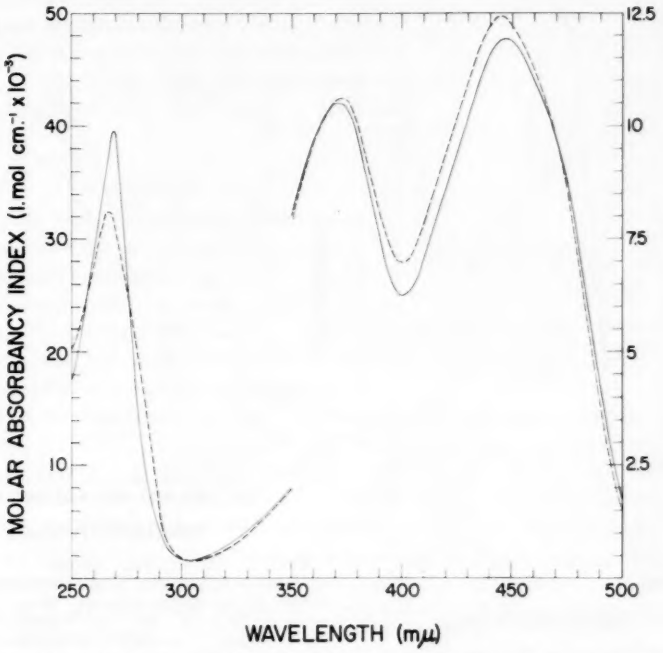

FIG. 1*a*.—Absorption spectra of 3-methylriboflavin and riboflavin in water solution. Cary model 11 spectrophotometer. *Full line*, 3-methylriboflavin; *dashed line*, riboflavin (see Whitby, *Biochem. J.*, **54**, 437, 1953).

Methods.—The visible and near-ultraviolet absorption maxima of water solutions containing flavin in the presence of the interactants studied lie at slightly different wave lengths and are of somewhat lower intensity than the corresponding maxima of water solutions of the *iso*alloxazine compounds alone. This is illustrated in Figure 2. Similar observations have been reported previously by others.[16–19] If it is assumed that this shift reflects complex formation, then apparent dissociation constants may be calculated. Assuming an equilibrium, $F + I \rightleftharpoons FI$, the following relationship obtains at fixed wave length when $[I]_0 \gg [F]_0$:

$$\frac{1}{\Delta A_s} = \frac{1}{[(a_M)_{FI} - (a_M)_F]l[F]_0} + \frac{K_c'}{[(a_M)_{FI} - (a_M)_F]l[F]_0} \cdot \frac{1}{[I]_0},$$

where F represents flavin, I = interactant, FI = a 1:1 complex of flavin and interactant, $[I]_0$ = total molar concentration of interactant, $[F]_0$ = total molar concentration of flavin, $K_c' = [F]\,[I]/[FI]$, l = optical path length of solution, $(a_M)_F$ = molar absorbancy index of flavin, $(a_M)_{FI}$ = molar absorbancy index of complex, A_s = absorbancy, and ΔA_s = (A_s for a solution of F at concentration $[F]_0$) + (A_s for a solution of I at concentration $[I]_0$) − (A_s for a solution of F, I, and FI in equilibrium at a total flavin concentration $[F]_0$ and a total interactant concentration $[I]_0$). Values for K_c' can conveniently be determined graphically from plots of $1/\Delta A_s$ versus $1/[I]_0$, at fixed values of $[F]_0$. Calculations were made for wave

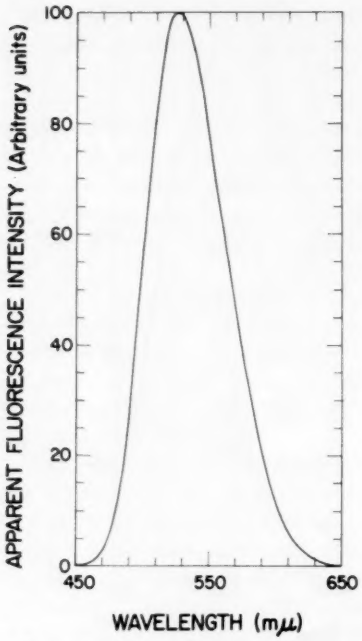

FIG. 1b.—Apparent fluorescence spectrum of 3-methylriboflavin, 1×10^{-5} M solution in water. See text for instrumentation. No corrections applied.

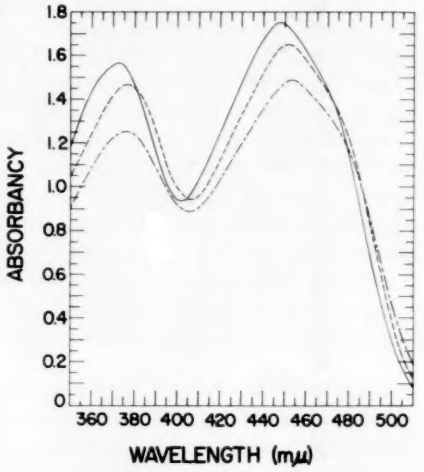

FIG. 2.—Absorption spectra of solutions of 3-methylriboflavin plus interactant. Cary model 11 spectrophotometer, 10-cm. cuvettes. ———, 1.5×10^{-5} M 3-methylriboflavin versus water; – – –, 1.5×10^{-5} M 3-methylriboflavin plus 0.21 M benzoate versus 0.21 M benzoate; – · –, 1.5×10^{-5} M 3-methylriboflavin plus 0.020 M 2-naphthoate versus 0.020 M 2-naphthoate.

lengths near 445 mμ and often also near 373 mμ. The method becomes decreasingly sensitive with increasing degree of dissociation.

Spectra were determined for solutions containing flavin at total concentrations from 1.0×10^{-5} M to 1.5×10^{-4} M, and interactant at total concentrations from 1.1×10^{-3} M to 3.8×10^{-1} M. Cuvettes with 1-cm. and 10-cm. path lengths were employed. Solutions were adjusted with potassium hydroxide or hydrochloric acid to values of pH between 6.5 and 7.5, but no buffers were added. In preliminary experiments with several interactants, no effect of pH on degree of interaction was observed over the range of pH 4–8. All measurements were made at 22.5° ± 0.5° C.

Results.—The values of ΔA_s are not large, and the range of concentrations of interactant that could be studied in water solution was limited, but the results obtained suffice for the immediate purpose of evaluating gross relationships. A representative graph of $1/\Delta A_s$ versus $1/[I]_0$ is shown in Figure 3. Within the range of concentrations employed, the assumed 1:1 stoichiometry represents the simplest formulation consistent with the data at hand. Apparent equilibrium constants are summarized in Table 1. The value of 0.021 mol $l.^{-1}$ found for the apparent dissociation constant of the 3-methylriboflavin-caffeine system at 22.5° C. (Fig. 3) may be compared with a value of 0.011 mol $l.^{-1}$ for riboflavin-caffeine at 17° C. and pH 7.5, determined fluorometrically by Weber,[16] and a value of 0.019 mol $l.^{-1}$ for riboflavin-caffeine at 20° C. and pH 5.8, determined spectrophotometrically by Yagi and Matsuoka.[18]

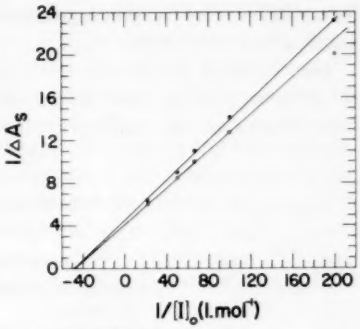

FIG. 3.—3-Methylriboflavin-caffeine system. *Solid circles*, readings at 373 mμ; *open circles*, readings at 445 mμ.

DISCUSSION

Currently accepted concepts of the mode of linkage between *iso*alloxazine and protein are essentially variations and extensions of views first advanced by Kuhn and his coworkers,[3, 20] who suggested that, in the "old yellow enzyme," a bond exists between the protein and the imino group in the number 3 position of the *iso*alloxazine ring. This suggestion was based upon the following observations. First, the fluorescence of FMN is quenched on dissociation of the number 3 imino group in alkaline solution[21] and also on linkage of FMN to the protein of the "old yellow enzyme."[1, 2] Furthermore, neither 3-methylriboflavin nor 3 methyl FMN can react with the protein of the "old yellow enzyme" to form an enzymically active complex,[3] and 3-methylriboflavin displays no growth-promoting activity upon administration to rats on a riboflavin-free diet.[22] A number of reviews state, as additional argument, that 3-methylriboflavin is nonfluorescent, but this is incorrect (Fig. 1).

During the past decade, considerable support has developed for the view that the interaction between protein and *iso*alloxazine rests on hydrogen bonding. Geissman[23] has proposed that perhaps not only the number 3 ring position but also the number 10 position participates in bonding to the protein and that both linkages involve hydrogen bonds to peptide chain —NH—CO— groups. Hydrogen-bond formation to the number 10 ring nitrogen also has been postulated by Michaelis.[24] More recently, Theorell and Nygaard have performed kinetic studies with both native and iodinated "old yellow enzyme" and have interpreted their data in terms of hydrogen-bonding effects.[4, 5] Extending a suggestion by Weber[16] that aromatic constituents such as tyrosine are likely participants in the quenching of flavin fluorescence by proteins, they have proposed that in the "old yellow enzyme" the number 3 imino group of the *iso*alloxazine ring is linked through a hydrogen bond to the OH group of a tyrosine residue.

Similar suggestions have been advanced for systems composed of riboflavin and

molecules other than proteins. Sakai[19] has investigated the effects of a number of aromatic compounds on the fluorescence of riboflavin and its solubility in water and has interpreted his results in terms of hydrogen-bond formation involving the imino or carbonyl groups of the *iso*alloxazine ring. Yagi and Matsuoka,[18] in a study of the reaction of riboflavin with phenol, advanced a similar hypothesis. Weber[16] has concluded that the quenching of the fluorescence of riboflavin by purines is due to complex formation and has suggested that in flavin adenine dinucleotide an internal complex exists between the *iso*alloxazine and adenine components of the molecule. His data have been interpreted[25] as indicating that bond formation occurs in flavin adenine dinucleotide between *iso*alloxazine and the amino group of adenine.

The results of the present study support a different point of view. In several of the systems studied, there is no possibility of hydrogen-bond formation between the interactant and the *iso*alloxazine ring. An example is 3-methylriboflavin-caffeine.

TABLE 1

APPARENT DISSOCIATION CONSTANTS

K_c' (MOL L.$^{-1}$ × 10^2)	INTERACTANT	FLAVIN*		
		FMN	Riboflavin	3-Methyl-riboflavin
1–5	3-Hydroxy-2-naphthoate	x	x	x
5–10	2-Naphthoate	x	x	x
	Anthraquinone-1-sulfonate	x		
	Anthraquinone-2,6-disulfonate	x		
10–50	*p*-Methoxycinnamate	x		
	p-Hydroxycinnamate	x		
	Cinnamate	x	x	x
	L-Tryptophan	x		x
	Caffeine	x	x	x
>50	Anisate	x	x	x
	p-Hydroxybenzoate	x	x	x
	Salicylate	x		
	m-Chlorophenol	x		
	Phenol	x		
	L-Tyrosine ethyl ester		x	x
	Benzoate	x	x	x
	L-Phenylalanine			x
	1,4-Benzoquinone	x		

* x marks denote systems studied.

Among those systems where hydrogen bonding is possible in principle, the relative values of the apparent dissociation constants recorded bear little relation to what would be predicted, were the interaction dependent upon hydrogen-bond formation alone. The constants recorded depend greatly upon the extent of conjugation of the compounds employed, and 3-methylriboflavin has in all instances proved fully as reactive as riboflavin or FMN. These observations argue against ring hydrogen bonding as a primary feature of interaction in the *iso*alloxazine systems examined and suggest that molecular charge transfer[26, 27] may be a more important factor. However, other interpretations may be possible, and definitive evidence in favor of charge-transfer interaction remains to be obtained. Kosower[28] previously has discussed the possibility that certain diphosphopyridine nucleotide systems might be charge-transfer complexes.

The choice of compounds included in this initial survey was rather arbitrary and was influenced by considerations of ready availability, solubility, and con-

venience of spectrophotometric observation. Nevertheless, the interactants listed in Table 1 include representatives of aromatic amino acids and purines, as well as compounds structurally related to certain vitamins, hormones, and agents known to uncouple electron transport from oxidative phosphorylation. It is tempting to speculate on the role which charge-transfer interaction and resonance coupling[29-32] may play in the prosthetic group-linked functions of flavoproteins and other oxidative enzymes. Interesting possibilities suggest themselves in relation to electron transport-coupled phenomena such as oxidative phosphorylation. However, such speculation would be premature. Whether the data presented have relevance to the interactive effects which obtain in *iso*alloxazine-protein systems is still to be established.

SUMMARY

Interaction in water solution between *iso*alloxazine derivatives and various conjugated molecules has been studied. The results obtained argue against hydrogen bonding between interactant and *iso*alloxazine as a primary feature of interaction in the systems examined and suggest that molecular charge transfer may be a more important factor. Alternative interpretations are possible. The data are discussed in relation to current concepts of the interaction of *iso*alloxazine with proteins and with other compounds of biochemical interest.

* This study was aided by grants from the National Science Foundation, the Rockefeller Foundation, and the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

† Predoctoral Research Fellow of the United States Public Health Service.

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PRODUCTION, REPRODUCTION, AND REVERSION OF PROTOPLAST-LIKE STRUCTURES IN THE OSMOTIC STRAIN OF NEUROSPORA CRASSA

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Communicated by G. W. Beadle, May 16, 1958

Protoplasts devoid of cell walls have been produced in *Bacillus megaterium* and certain other Gram-positive species of bacteria.[1] Structures resembling protoplasts but not completely devoid of cell walls have also been produced in *Escherichia coli* and some other Gram-negative bacteria.[2] Those from Gram-positive and Gram-negative bacteria are alike in that they are spherical in shape and are lysed by osmotic shock. Bacterial protoplasts have already proved to have many useful applications—among others, in the extraction of cellular constituents, in studies of the biosynthesis of enzymes and other macromolecular substances, and in host-parasite interrelations.[3]

Protoplasts, or protoplast-like structures, have also been produced in one of the higher fungi, the unicellular Ascomycete, *Saccharomyces cerevisiae*.[4] The present paper reports the production and reproduction of coenocytic protoplast-like structures in one strain of the filamentous Ascomycete, *Neurospora crassa*.

Strain Specificity.—Under the conditions employed, protoplasts have consistently been formed by all tested strains carrying the osmotic mutant gene, *os*.[5] An exhaustive survey has not been made, but all tested strains carrying the wild-type allele of *os* have either been completely refractory or have responded poorly. The only non-osmotic strains which have so far yielded protoplasts are two with maternally inherited cytochrome abnormalities, *poky*[6] and *mi-3*,[7] which appear to require higher enzyme concentrations than strains carrying *os* and, even then, are erratic in response. Descriptions to follow refer to *os* strains.

Treatment.—Culture media in which protoplasts have been produced consist of the standard *Neurospora* salt mixture and biotin,[8] sugars, and either a commercial hemicellulase preparation[9] or, in a few tests, a crude preparation of snail hepatic juice.

While the concentrations of enzymes, salts, and sugars do influence the ease with which protoplasts are produced and maintained, no clear end-point for dilution

has been observed in either their production or their maintenance. Protoplasts have been produced in media containing as little as 0.5 per cent (*w/v*) hemicellulase, 1 per cent sucrose, and the standard salt mixture. In most experiments, however, media have contained 2 or 3 per cent hemicellulase, 2 per cent sucrose, 5–10 per cent rhamnose or sorbose, and two to four times the standard strength of the salt mixture. Protoplasts are more readily maintained at the higher concentrations of sugars and enzymes and are more uniformly lysed by osmotic shock than those cultured at lower concentrations.

Origin, Growth, and Reproduction of Protoplasts.—When hyphae or hyphal fragments are transferred to hemicellulase media, protoplasts are extruded through pores in the side or end walls of the hyphal cells. Monilioid conidia (macroconidia)

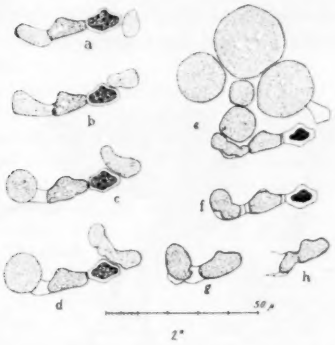

FIG. 1.—Camera lucida drawings of successive stages in protoplast development from two of three conidia; the conidium in the middle is degenerating. Conidia of osmotic strain Em 11200a in 10 per cent rhamnose, 2 per cent sucrose, 3 per cent hemicellulase concentrate, 3.2× standard salt mixture, in a small drop under oil at room temperature. Elapsed times in hours and minutes: inoculation to *a*, 4:15; *a* to *b*, 0:25; *b* to *c*, 1:30; *c* to *d*, 1:25; *d* to *e*, 13:30; *e* to *f*, 0:13; *f* to *g*, 0:30; *g* to *h*, 0:20.

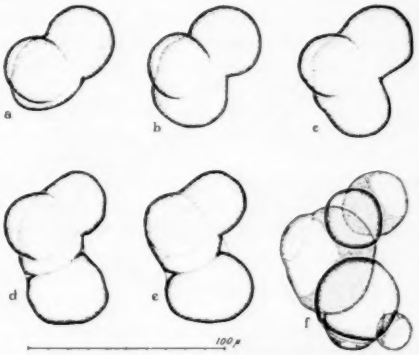

FIG. 2.—Growth of a protoplast subcultured in 10 per cent sorbose, 2 per cent sucrose, 2 per cent hemicellulase concentrate, 4× standard salt mixture, in a small drop under oil at room temperature. Elapsed times: inoculation to *a*, 20:05; *a* to *b*, 1:37; *b* to *c*, 1:48; *c* to *d*, 1:05; *d* to *e*, 0:50; *e* to *f*, 21:15.

germinate directly into protoplasts (Fig. 1). Protoplasts produced directly from conidia seem to be less uniform than those arising from hyphae, especially at low concentrations of sugars and enzymes, and are more likely to revert to hyphal-type growth in the media in which they were produced.

Newly formed protoplasts are spherical, average about 10 μ in diameter, and contain several nuclei. Most protoplasts increase in diameter to about 50 μ, some to 100 μ, and contain a hundred or more nuclei. Upon lysis in distilled water, the cell contents disperse rapidly, leaving delicate membranes, or cell walls, the constitution of which has not been determined. Protoplasts produced by snail enzymes are also readily lysed by osmotic shock, but much more prominent walls remain.

In older cultures many protoplasts become highly vacuolate. When these are

lysed, the discharged vacuoles remain visible and increase in diameter to as much as 50 μ without bursting. Membranes of discharged vacuoles show remarkable elasticity when poked with fine glass needles. Upon transfer to fresh media, highly vacuolated protoplasts continue growth and division.

Division of protoplasts commonly occurs as a simple pinching in two by a furrowing process (Fig. 2) or sometimes by a process resembling the budding of yeasts (similar to that illustrated in Fig. 3). Daughter protoplasts are at first contained within the membrane of the parental cell, but they separate readily, either spontaneously or upon gentle agitation.

When protoplasts are grown on an agar surface or on glass, they often become greatly flattened, irregularly shaped, and closely packed into a thin sheet (similar to the nonreverted cells in Fig. 4). Division of protoplasts under these conditions continues by rather irregular furrowing. These sheets of protoplasts resemble the multinucleate protoplasts of yeasts figured by Nečas,[4] and the L-forms of *E. coli* described by Lederberg.[10]

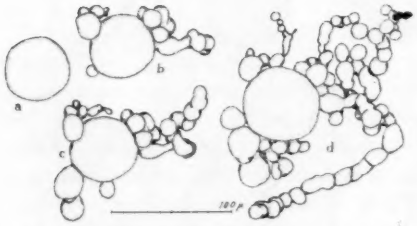

FIG. 3.—Successive stages in the reversion of a protoplast to hyphal-type growth after transfer from hemicellulase medium to 10 per cent sorbose, 2 per cent sucrose, 4× standard salt mixture (osmotic concentration 30.7 atm.), in 0.2 per cent agar under oil at room temperature. Elapsed times: inoculation to *a*, 0:15; *a* to *b*, 6:23; *b* to *c*, 2:22; *c* to *d*, 11:50.

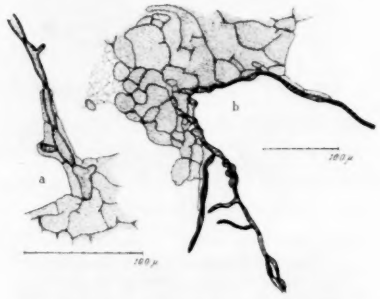

FIG. 4.—Portions of a flat pseudo-parenchymatous colony developed from protoplasts on an inverted cover-glass mount, showing transition from parenchymatous to hyphal growth, about 48 hours after transfer from a hemicellulase medium to one containing 5 per cent sucrose and the standard salt mixture (osmotic concentration 7.5 atm.).

Reversion to Hyphal-Type Growth.—Upon transfer of protoplasts to media lacking enzymes, they revert to typical mycelial growth, though the time necessary for complete reversion varies from a few hours to several days. The type of mycelial growth produced by *os* strains depends upon the osmotic strength of the medium.[5] In media of less than 5 atm. osmotic pressure, long, thin, sparsely branched hyphae are produced (as in Fig. 4). At higher osmotic concentrations, there result progressively more contorted and more branched hyphae, with short, broad cells (Fig. 3). Individual protoplasts sometimes produce one or more hyphae as direct outgrowths of the mother cell. In other instances (as in Figs. 3 and 4) a considerable amount of parenchymatous tissue is first formed, from which hyphae differentiate. Variations in response to the absence of enzymes, in respect to both the time required for reversion and the growth forms produced, may reflect the degree of somatic differentiation of individual protoplasts from hyphal-type growth, as well as differences in the cultural conditions under which they had developed.

Appearances of regeneration from naked masses of protoplasm have sometimes been noted, but never in examples in which it had been established that there were no small, fully formed cells from which the growth might have arisen. It is, of course, possible that cultural conditions will be found under which masses of naked protoplasm will revert to cellular growth, such as has been reported by Nečas[4] in *Saccharomyces cerevisiae* and by Pease[11] in *Proteus vulgaris*.

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*THE REPLICATION OF DNA IN ESCHERICHIA COLI**

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Communicated by Max Delbrück, May 14, 1958

Introduction.—Studies of bacterial transformation and bacteriaphage infection[1–5] strongly indicate that deoxyribonucleic acid (DNA) can carry and transmit hereditary information and can direct its own replication. Hypotheses for the mechanism of DNA replication differ in the predictions they make concerning the distribution among progeny molecules of atoms derived from parental molecules.[6]

Radioisotopic labels have been employed in experiments bearing on the distribution of parental atoms among progeny molecules in several organisms.[6–9] We anticipated that a label which imparts to the DNA molecule an increased density might permit an analysis of this distribution by sedimentation techniques. To this end, a method was developed for the detection of small density differences among

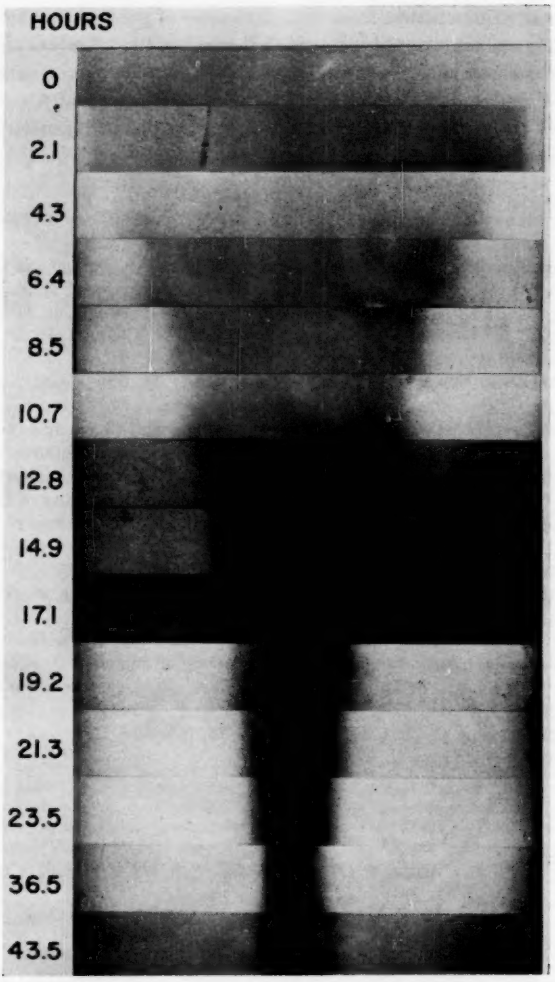

FIG. 1.—Ultraviolet absorption photographs showing successive stages in the banding of DNA from *E. coli*. An aliquot of bacterial lysate containing approximately 10^8 lysed cells was centrifuged at 31,410 rpm in a CsCl solution as described in the text. Distance from the axis of rotation increases toward the right. The number beside each photograph gives the time elapsed after reaching 31,410 rpm.

macromolecules.[10] By use of this method, we have observed the distribution of the heavy nitrogen isotope N^{15} among molecules of DNA following the transfer of a uniformly N^{15}-labeled, exponentially growing bacterial population to a growth medium containing the ordinary nitrogen isotope N^{14}.

Density-Gradient Centrifugation.—A small amount of DNA in a concentrated solution of cesium chloride is centrifuged until equilibrium is closely approached

The opposing processes of sedimentation and diffusion have then produced a stable concentration gradient of the cesium chloride. The concentration and pressure gradients result in a continuous increase of density along the direction of centrifugal force. The macromolecules of DNA present in this density gradient are driven by the centrifugal field into the region where the solution density is equal to their own buoyant density.[11] This concentrating tendency is opposed by diffusion, with the result that at equilibrium a single species of DNA is distributed over a band whose width is inversely related to the molecular weight of that species (Fig. 1).

If several different density species of DNA are present, each will form a band at the position where the density of the CsCl solution is equal to the buoyant density of that species. In this way DNA labeled with heavy nitrogen (N^{15}) may be

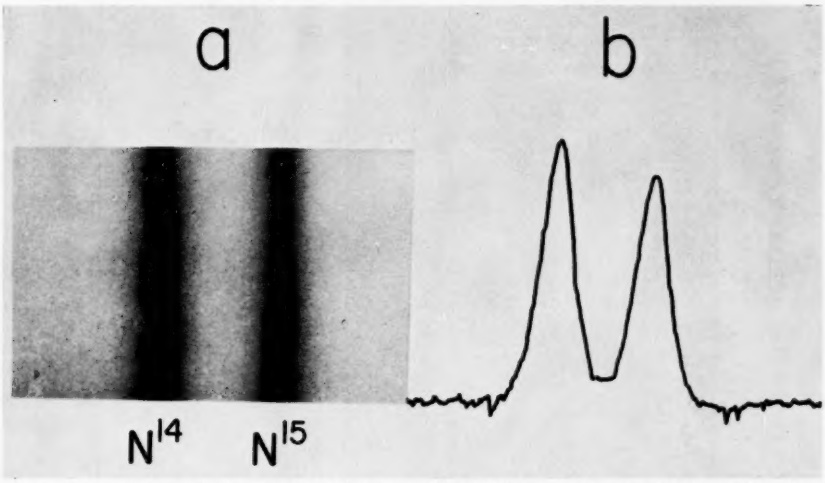

FIG. 2—*a:* The resolution of N^{14} DNA from N^{15} DNA by density-gradient centrifugation. A mixture of N^{14} and N^{15} bacterial lysates, each containing about 10^8 lysed cells, was centrifuged in CsCl solution as described in the text. The photograph was taken after 24 hours of centrifugation at 44,770 rpm. *b:* A microdensitometer tracing showing the DNA distribution in the region of the two bands of Fig. 2*a*. The separation between the peaks corresponds to a difference in buoyant density of 0.014 gm. $cm.^{-3}$

resolved from unlabeled DNA. Figure 2 shows the two bands formed as a result of centrifuging a mixture of approximately equal amounts of N^{14} and N^{15} *Escherichia coli* DNA.

In this paper reference will be made to the apparent molecular weight of DNA samples determined by means of density-gradient centrifugation. A discussion has been given[10] of the considerations upon which such determinations are based, as well as of several possible sources of error.[12]

Experimental.—Escherichia coli B was grown at 36° C. with aeration in a glucose salts medium containing ammonium chloride as the sole nitrogen source.[13] The growth of the bacterial population was followed by microscopic cell counts and by colony assays (Fig. 3).

Bacteria uniformly labeled with N^{15} were prepared by growing washed cells for

14 generations (to a titer of 2×10^8/ml) in medium containing 100 μg/ml of $N^{15}H_4Cl$ of 96.5 per cent isotopic purity. An abrupt change to N^{14} medium was then accomplished by adding to the growing culture a tenfold excess of $N^{14}H_4Cl$, along with ribosides of adenine and uracil in experiment 1 and ribosides of adenine, guanine, uracil, and cytosine in experiment 2, to give a concentration of 10 μg/ml of each riboside. During subsequent growth the bacterial titer was kept between

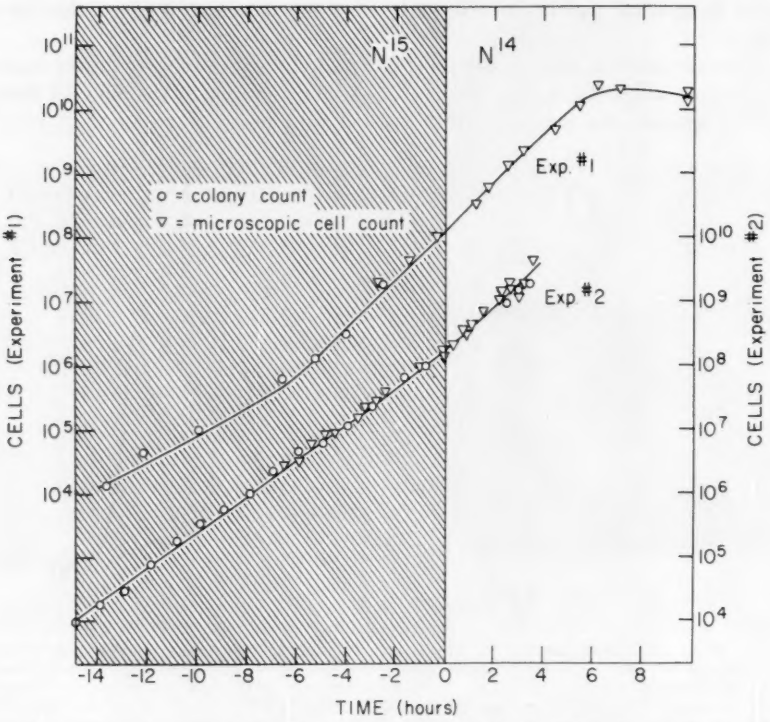

FIG. 3.—Growth of bacterial populations first in N^{15} and then in N^{14} medium. The values on the ordinates give the actual titers of the cultures up to the time of addition of N^{14}. Thereafter, during the period when samples were being withdrawn for density-gradient centrifugation, the actual titer was kept between 1 and 2×10^8 by additions of fresh medium. The values on the ordinates during this later period have been corrected for the withdrawals and additions. During the period of sampling for density-gradient centrifugation, the generation time was 0.81 hours in Experiment 1 and 0.85 hours in Experiment 2.

1 and 2×10^8/ml by appropriate additions of fresh N^{14} medium containing ribosides.

Samples containing about 4×10^9 bacteria were withdrawn from the culture just before the addition of N^{14} and afterward at intervals for several generations. Each sample was immediately chilled and centrifuged in the cold for 5 minutes at 1,800$\times$ g. After resuspension in 0.40 ml. of a cold solution 0.01 M in NaCl and 0.01 M in ethylenediaminetetra-acetate (EDTA) at pH 6, the cells were lysed by the addition of 0.10 ml. of 15 per cent sodium dodecyl sulfate and stored in the cold.

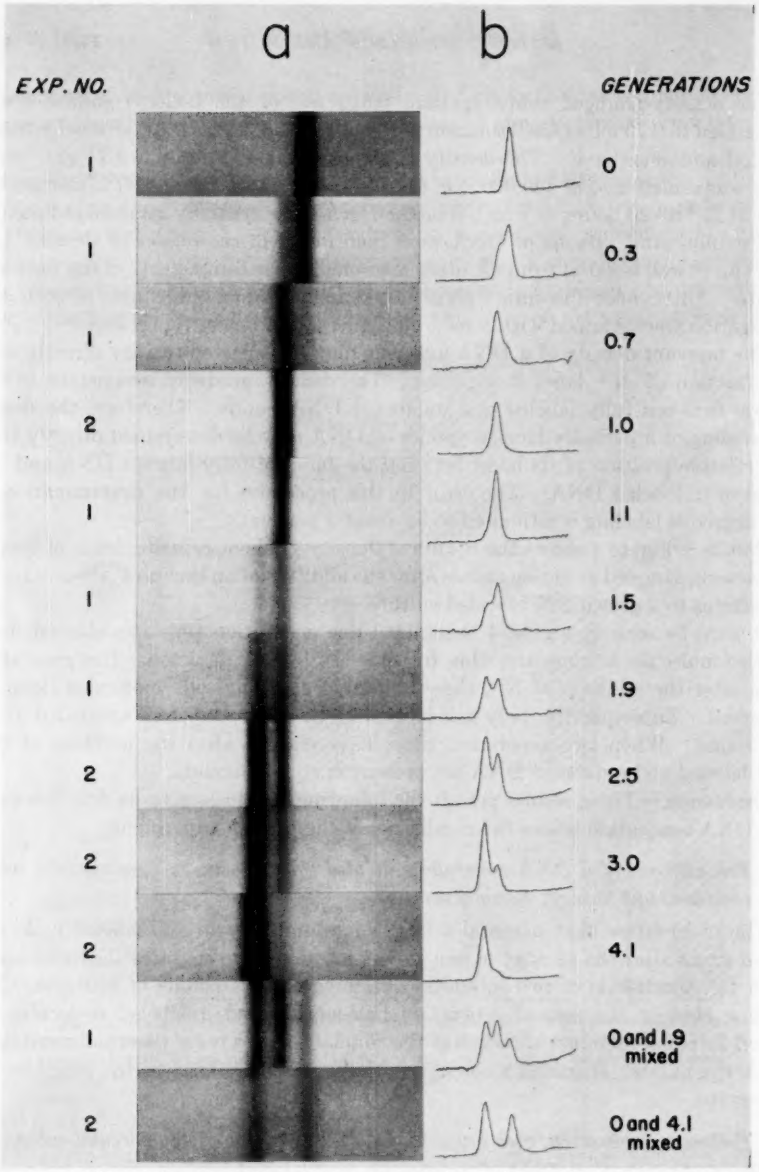

FIG. 4—*a:* Ultraviolet absorption photographs showing DNA bands resulting from density-gradient centrifugation of lysates of bacteria sampled at various times after the addition of an excess of N^{14} substrates to a growing N^{15}-labeled culture. Each photograph was taken after 20 hours of centrifugation at 44,770 rpm under the conditions described in the text. The density of the CsCl solution increases to the right. Regions of equal density occupy the same horizontal position on each photograph. The time of sampling is measured from the time of the addition of N^{14} in units of the generation time. The generation times for Experiments 1 and 2 were estimated from the measurements of bacterial growth presented in Fig. 3. *b:* Microdensitometer tracings of the DNA bands shown in the adjacent photographs. The microdensitometer pen displacement above the base line is directly proportional to the concentration of DNA. The degree of labeling of a species of DNA corresponds to the relative position of its band between the bands of fully labeled and unlabeled DNA shown in the lowermost frame, which serves as a density reference. A test of the conclusion that the DNA in the band of intermediate density is just half-labeled is provided by the frame showing the mixture of generations 0 and 1.9. When allowance is made for the relative amounts of DNA in the three peaks, the peak of intermediate density is found to be centered at 50 ± 2 per cent of the distance between the N^{14} and N^{15} peaks.

For density-gradient centrifugation, 0.010 ml. of the dodecyl sulfate lysate was added to 0.70 ml. of CsCl solution buffered at pH 8.5 with 0.01 *M* tris(hydroxymethyl)aminomethane. The density of the resulting solution was 1.71 gm. cm.$^{-3}$ This was centrifuged at 140,000× *g*. (44,770 rpm) in a Spinco model E ultracentrifuge at 25° for 20 hours, at which time the DNA had essentially attained sedimentation equilibrium. Bands of DNA were then found in the region of density 1.71 gm. cm.$^{-3}$, well isolated from all other macromolecular components of the bacterial lysate. Ultraviolet absorption photographs taken during the course of each centrifugation were scanned with a recording microdensitometer (Fig. 4).

The buoyant density of a DNA molecule may be expected to vary directly with the fraction of N^{15} label it contains. The density gradient is constant in the region between fully labeled and unlabeled DNA bands. Therefore, the degree of labeling of a partially labeled species of DNA may be determined directly from the relative position of its band between the band of fully labeled DNA and the band of unlabeled DNA. The error in this procedure for the determination of the degree of labeling is estimated to be about 2 per cent.

Results.—Figure 4 shows the results of density-gradient centrifugation of lysates of bacteria sampled at various times after the addition of an excess of N^{14}-containing substrates to a growing N^{15}-labeled culture.

It may be seen in Figure 4 that, until one generation time has elapsed, half-labeled molecules accumulate, while fully labeled DNA is depleted. One generation time after the addition of N^{14}, these half-labeled or "hybrid" molecules alone are observed. Subsequently, only half-labeled DNA and completely unlabeled DNA are found. When two generation times have elapsed after the addition of N^{14}, half-labeled and unlabeled DNA are present in equal amounts.

Discussion.—These results permit the following conclusions to be drawn regarding DNA replication under the conditions of the present experiment.

1. *The nitrogen of a DNA molecule is divided equally between two subunits which remain intact through many generations.*

The observation that parental nitrogen is found only in half-labeled molecules at all times after the passage of one generation time demonstrates the existence in each DNA molecule of two subunits containing equal amounts of nitrogen. The finding that at the second generation half-labeled and unlabeled molecules are found in equal amounts shows that the number of surviving parental subunits is twice the number of parent molecules initially present. That is, the subunits are conserved.

2. *Following replication, each daughter molecule has received one parental subunit.*

The finding that all DNA molecules are half-labeled one generation time after the addition of N^{14} shows that each daughter molecule receives one parental subunit.[14] If the parental subunits had segregated in any other way among the daughter molecules, there would have been found at the first generation some fully labeled and some unlabeled DNA molecules, representing those daughters which received two or no parental subunits, respectively.

3. *The replicative act results in a molecular doubling.*

This statement is a corollary of conclusions 1 and 2 above, according to which each parent molecule passes on two subunits to progeny molecules and each progeny

molecule receives just one parental subunit. It follows that each single molecular reproductive act results in a doubling of the number of molecules entering into that act.

The above conclusions are represented schematically in Figure 5.

The Watson-Crick Model.—A molecular structure for DNA has been proposed by Watson and Crick.[15] It has undergone preliminary refinement[16] without alteration of its main features and is supported by physical and chemical studies.[17] The structure consists of two polynucleotide chains wound helically about a common axis. The nitrogen base (adenine, guanine, thymine, or cytosine) at each level

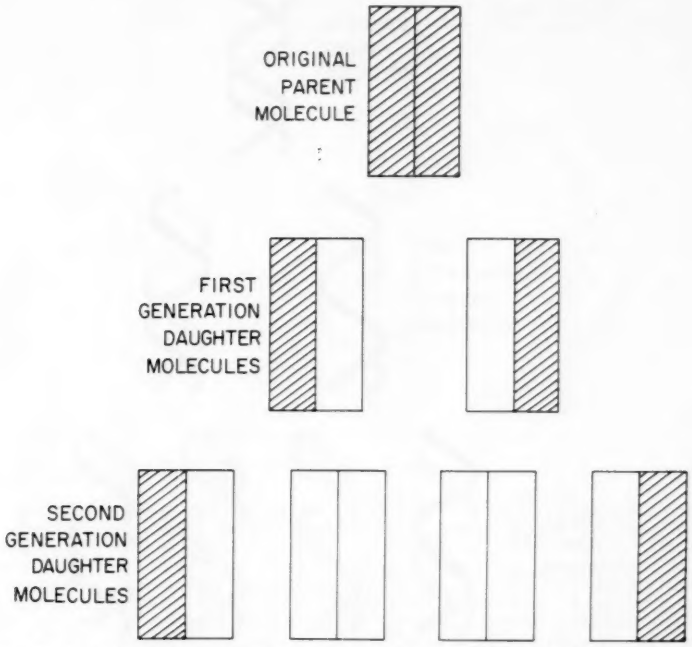

FIG. 5.—Schematic representation of the conclusions drawn in the text from the data presented in Fig. 4. The nitrogen of each DNA molecule is divided equally between two subunits. Following duplication, each daughter molecule receives one of these. The subunits are conserved through successive duplications.

on one chain is hydrogen-bonded to the base at the same level on the other chain. Structural requirements allow the occurrence of only the hydrogen-bonded base pairs adenine-thymine and guanine-cytosine, resulting in a detailed complementariness between the two chains. This suggested to Watson and Crick[18] a definite and structurally plausible hypothesis for the duplication of the DNA molecule. According to this idea, the two chains separate, exposing the hydrogen-bonding sites of the bases. Then, in accord with the base-pairing restrictions, each chain serves as a template for the synthesis of its complement. Accordingly, each daughter molecule contains one of the parental chains paired with a newly synthesized chain (Fig. 6).

The results of the present experiment are in exact accord with the expectations of the Watson-Crick model for DNA duplication. However, it must be emphasized that it has not been shown that the molecular subunits found in the present experiment are single polynucleotide chains or even that the DNA molecules studied here correspond to single DNA molecules possessing the structure proposed by Watson and Crick. However, some information has been obtained about the molecules and their subunits; it is summarized below.

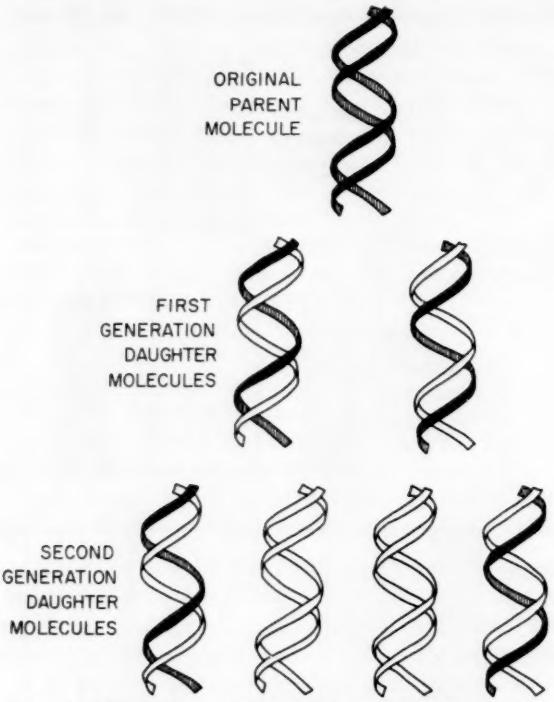

FIG. 6.—Illustration of the mechanism of DNA duplication proposed by Watson and Crick. Each daughter molecule contains one of the parental chains (*black*) paired with one new chain (*white*). Upon continued duplication, the two original parent chains remain intact, so that there will always be found two molecules each with one parental chain.

The DNA molecules derived from *E. coli* by detergent-induced lysis have a buoyant density in CsCl of 1.71 gm. cm.$^{-3}$, in the region of densities found for T2 and T4 bacteriophage DNA, and for purified calf-thymus and salmon-sperm DNA. A highly viscous and elastic solution of N^{14} DNA was prepared from a dodecyl sulfate lysate of *E. coli* by the method of Simmons[19] followed by deproteinization with chloroform. Further purification was accomplished by two cycles of preparative density-gradient centrifugation in CsCl solution. This purified bacterial DNA was found to have the same buoyant density and apparent molecular weight, 7×10^6, as the DNA of the whole bacterial lysates (Figs. 7, 8).

Heat Denaturation.—It has been found that DNA from *E. coli* differs importantly from purified salmon-sperm DNA in its behavior upon heat denaturation.

Exposure to elevated temperatures is known to bring about an abrupt collapse of the relatively rigid and extended native DNA molecule and to make available for acid-base titration a large fraction of the functional groups presumed to be blocked by hydrogen-bond formation in the native structure.[19, 20, 21, 22] Rice and Doty[22] have reported that this collapse is not accompanied by a reduction in molecular weight as determined from light-scattering. These findings are corroborated by density-gradient centrifugation of salmon-sperm DNA.[23] When this material is

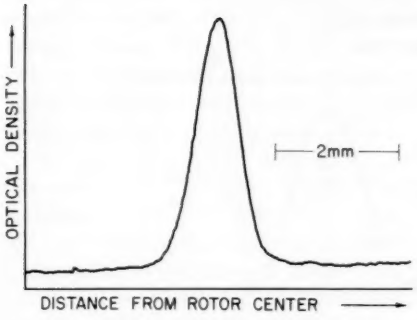

FIG. 7.—Microdensitometer tracing of an ultraviolet absorption photograph showing the optical density in the region of a band of N^{14} *E. coli* DNA at equilibrium. About 2 μg. of DNA purified as described in the text was centrifuged at 31,410 rpm at 25° in 7.75 molal CsCl at pH 8.4. The density gradient is essentially constant over the region of the band and is 0.057 gm./cm.^{4}. The position of the maximum indicates a buoyant density of 1.71 gm. cm.$^{-3}$ In this tracing the optical density above the base line is directly proportional to the concentration of DNA in the rotating centrifuge cell. The concentration of DNA at the maximum is about 50 μg./ml.

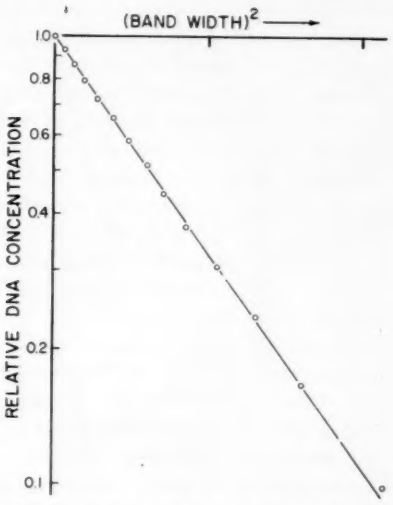

FIG. 8.—The square of the width of the band of Fig. 7 plotted against the logarithm of the relative concentration of DNA. The divisions along the abscissa set off intervals of 1 mm.^{2}. In the absence of density heterogeneity, the slope at any point of such a plot is directly proportional to the weight average molecular weight of the DNA located at the corresponding position in the band. Linearity of this plot indicates monodispersity of the banded DNA. The value of the the slope corresponds to an apparent molecular weight for the Cs-DNA salt of 9.4×10^6, corresponding to a molecular weight of 7.1×10^6 for the sodium salt.

kept at 100° for 30 minutes either under the conditions employed by Rice and Doty or in the CsCl centrifuging medium, there results a density increase of 0.014 gm. cm.$^{-3}$ with no change in apparent molecular weight. The same results are obtained if the salmon-sperm DNA is pre-treated at pH 6 with EDTA and sodium dodecyl sulfate. Along with the density increase, heating brings about a sharp reduction in the time required for band formation in the CsCl gradient. In the absence of an increase in molecular weight, the decrease in banding time must be ascribed[10] to an increase in the diffusion coefficient, indicating an extensive collapse of the native structure.

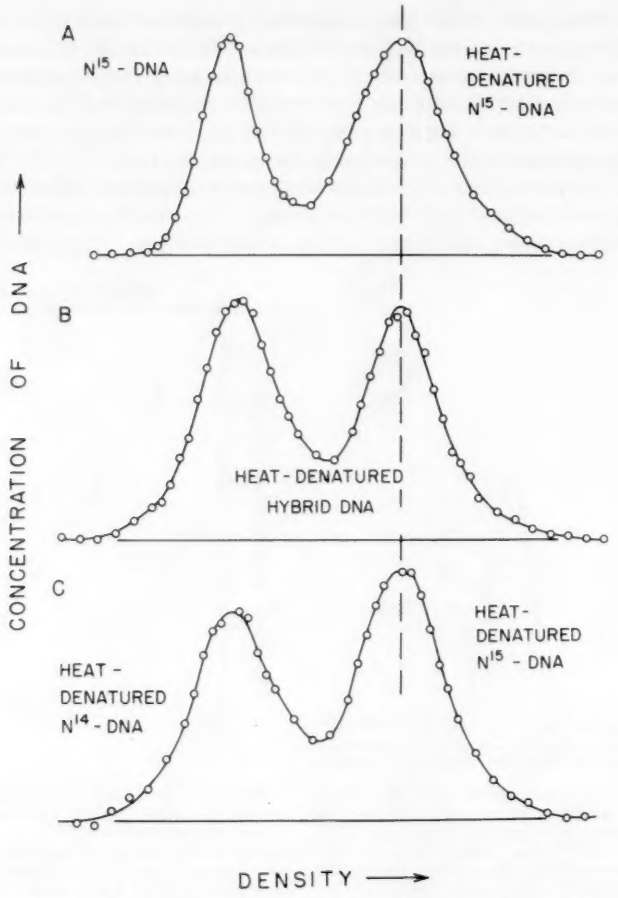

FIG. 9.—The dissociation of the subunits of *E. coli* DNA upon heat denaturation. Each smooth curve connects points obtained by microdensitometry of an ultraviolet absorption photograph taken after 20 hours of centrifugation in CsCl solution at 44,770 rpm. The baseline density has been removed by subtraction. *A:* A mixture of heated and unheated N^{15} bacterial lysates. Heated lysate alone gives one band in the position indicated. Unheated lysate was added to this experiment for comparison. Heating has brought about a density increase of 0.016 gm. cm.$^{-3}$ and a reduction of about half in the apparent molecular weight of the DNA. *B:* Heated lysate of N^{15} bacteria grown for one generation in N^{14} growth medium. Before heat denaturation, the hybrid DNA contained in this lysate forms only one band, as may be seen in Fig. 4. *C:* A mixture of heated N^{14} and heated N^{15} bacterial lysates. The density difference is 0.015 gm. cm.$^{-3}$

The decrease in banding time and a density increase close to that found upon heating salmon-sperm DNA are observed (Fig. 9, *A*) when a bacterial lysate containing uniformly labeled N^{15} or N^{14} *E. coli* DNA is kept at 100° C. for 30 minutes in the CsCl centrifuging medium; but the apparent molecular weight of

the heated bacterial DNA is reduced to approximately half that of the unheated material.

Half-labeled DNA contained in a detergent lysate of N^{15} *E. coli* cells grown for one generation in N^{14} medium was heated at 100° C. for 30 minutes in the CsCl centrifuging medium. This treatment results in the loss of the original half-labeled material and in the appearance in equal amounts of two new density species, each with approximately half the initial apparent molecular weight (Fig. 9, *B*). The density difference between the two species is 0.015 gm. cm.$^{-3}$, close to the increment produced by the N^{15} labeling of the unheated DNA.

This behavior suggests that heating the hybrid molecule brings about the dissociation of the N^{15}-containing subunit from the N^{14} subunit. This possibility was tested by a density-gradient examination of a mixture of heated N^{15} DNA and heated N^{14} DNA (Fig. 9, *C*). The close resemblance between the products of heating hybrid DNA (Fig. 9 *B*) and the mixture of products obtained from heating N^{14} and N^{15} DNA separately (Fig. 9, *C*) leads to the conclusion that the two molecular subunits have indeed dissociated upon heating. Since the apparent molecular weight of the subunits so obtained is found to be close to half that of the intact molecule, it may be further concluded that the subunits of the DNA molecule which are conserved at duplication are single, continuous structures. The scheme for DNA duplication proposed by Delbrück[24] is thereby ruled out.

To recapitulate, both salmon-sperm and *E. coli* DNA heated under similar conditions collapse and undergo a similar density increase, but the salmon DNA retains its initial molecular weight, while the bacterial DNA dissociates into the two subunits which are conserved during duplication. These findings allow two different interpretations. On the one hand, if we assume that salmon DNA contains subunits analogous to those found in *E. coli* DNA, then we must suppose that the subunits of salmon DNA are bound together more tightly than those of the bacterial DNA. On the other hand, if we assume that the molecules of salmon DNA do not contain these subunits, then we must concede that the bacterial DNA molecule is a more complex structure than is the molecule of salmon DNA. The latter interpretation challenges the sufficiency of the Watson-Crick DNA model to explain the observed distribution of parental nitrogen atoms among progeny molecules.

Conclusion.—The structure for DNA proposed by Watson and Crick brought forth a number of proposals as to how such a molecule might replicate. These proposals[6] make specific predictions concerning the distribution of parental atoms among progeny molecules. The results presented here give a detailed answer to the question of this distribution and simultaneously direct our attention to other problems whose solution must be the next step in progress toward a complete understanding of the molecular basis of DNA duplication. What are the molecular structures of the subunits of *E. coli* DNA which are passed on intact to each daughter molecule? What is the relationship of these subunits to each other in a DNA molecule? What is the mechanism of the synthesis and dissociation of the subunits in vivo?

Summary.—By means of density-gradient centrifugation, we have observed the distribution of N^{15} among molecules of bacterial DNA following the transfer of a uniformly N^{15}-substituted exponentially growing *E. coli* population to N^{14} medium.

We find that the nitrogen of a DNA molecule is divided equally between two physically continuous subunits; that, following duplication, each daughter molecule receives one of these; and that the subunits are conserved through many duplications.

* Aided by grants from the National Foundation for Infantile Paralysis and the National Institutes of Health.

† Contribution No. 2344.

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[11] The buoyant density of a molecule is the density of the solution at the position in the centrifuge cell where the sum of the forces acting on the molecule is zero.

[12] Our attention has been called by Professor H. K. Schachman to a source of error in apparent molecular weights determined by density-gradient centrifugation which was not discussed by Meselson, Stahl, and Vinograd. In evaluating the dependence of the free energy of the DNA component upon the concentration of CsCl, the effect of solvation was neglected. It can be shown that solvation may introduce an error into the apparent molecular weight if either CsCl or water is bound preferentially. A method for estimating the error due to such selective solvation will be presented elsewhere.

[13] In addition to NH_4Cl, this medium consists of 0.049 *M* Na_2HPO_4, 0.022 *M* KH_2PO_4, 0.05 *M* NaCl, 0.01 *M* glucose, 10^{-3} *M* $MgSO_4$, and 3×10^{-6} *M* $FeCl_2$.

[14] This result also shows that the generation time is very nearly the same for all DNA molecules in the population. This raises the questions of whether in any one nucleus all DNA molecules are controlled by the same clock and, if so, whether this clock regulates nuclear and cellular division as well.

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SIGNIFICANT STRUCTURES IN THE LIQUID STATE. I

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DEPARTMENT OF CHEMISTRY, UNIVERSITY OF UTAH

Communicated May 23, 1958

The mechanical properties of solids can be understood only by taking proper account of the imperfections present in the crystal lattice. Such lattice imperfections are so important in the liquid as to be an essential part of any description of this less ordered state. When a normal liquid such as argon melts, it expands 12 per cent. Such an expansion is typical. The atypical 10 per cent contraction of ice upon melting can be made to disappear if one first applies some 2,500-atm. pressure, at an appropriate temperature, to the hydrogen-bonded tetrahedral ice I and so transforms it into close-packed ice III, which is a fifth more dense. Ice III then melts with a normal 10 per cent expansion. Anhydrous salts such as sodium chloride melt with about the normal 10 per cent expansion. Metals such as sodium melt with about a 3 per cent expansion. This small expansion on melting of metals was long ago interpreted[1] as a consequence of the small size of the ion formed from atoms which no longer localize their conducting electrons. The ion, being only about one-third atomic size, requires only a third the expansion of normal liquids on melting to provide the same number of vacant sites and therefore the same increased disorder, which leads to melting.

To account for the properties of the liquid state in general and of melting in particular, we require a model. We shall describe our procedure for arriving at this model as the identification of significant structures. A simple example will clarify the procedure.

Suppose we wish to calculate the thermodynamics of iodine in the gaseous state at a temperature at which the diatomic molecules are partly dissociated into atoms. Clearly, there are two significant structures—diatomic molecules and atoms—and the partition function for the whole is a product of partition functions for the molecules and for the atoms. Now, since a diatomic molecule and a monatomic one contribute equally to the pressure, the pressure at any temperature measures the total number of diatomic plus monatomic molecules per cubic centimeter. A knowledge of the density, together with the pressure, fixes the composition and thereby all thermodynamic properties. For example, the energy, like the pressure, follows immediately from the composition in the iodine system. The composition is fixed by the easily formulated requirement that molecules and atoms be at the same chemical potential. Knowing the composition, the partition function is expressible in terms of the independent variables V and T, so that all the thermodynamic properties of the system become readily calculable. The key to success in this procedure is the recognition of the two significant structures—iodine molecules and iodine atoms.

We proceed to develop the method of significant structures for the liquid state. Figure 1 shows a partial dislocation extending from top to bottom through the middle of the figure. The nine molecules to the left (west) of the dislocation occupy what we will call a sites, while the nine molecules on the right (east) occupy b sites. If the distance between centers of close-packed neighboring molecules be

indicated by d, one can see that molecules on a sites bordering the dislocation can shift to adjacent empty b sites by moving northeast a distance $d/\sqrt{3}$. Similarly, molecules on b sites next to the dislocation can move to empty neighboring a sites by proceeding southwest a distance $d/\sqrt{3}$. Such molecular displacements merely shift the position of dislocations without creating or destroying them. This shifting of molecular positions we call a-to-b transitions or b-to-a transitions, as the case may be, and write for them, as for chemical reactions, the expressions $a \rightarrow b$ and $b \rightarrow a$, respectively. When an $a \rightarrow b$ or $b \rightarrow a$ transition occurs, there is an elastic readjustment of all neighbors, with a net storage or release of energy.

Long, narrow passageways in glass like the dislocation in Figure 1 have long been known to exist. Thus Pyrex leaks helium, which has a diameter of 2 A and is impervious to H_2 with a diameter of 2.5 A as well as larger molecules. Soft glass

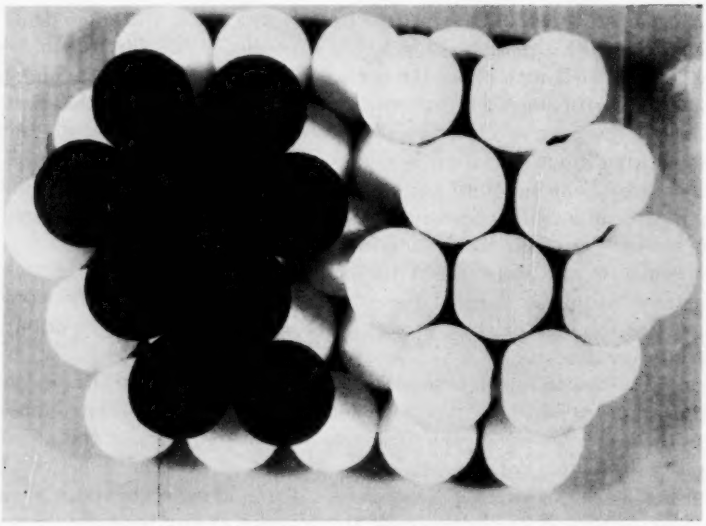

FIG. 1.—Model of type dislocations present in the liquid state.

has its passageways partly obstructed, as helium does not leak through it. Water containing sulfuric acid when frozen into glass and irradiated has been observed[2] to give hydrogen atoms at twenty times the yield obtained from crystalline materials similarly treated. Escape passageways in the glass would explain the difference. When sulfuric acid is replaced by phosphoric and some other acids, the differences in hydrogen yield almost disappear. Since a glass is presumably a metastable liquid with the passageways frozen in, it is to be expected that such passageways would be present in the liquid.

Thus we are led to identify three significant structures in the liquid state. The first structure is that of molecules with solid-like degrees of freedom. Thus in the direction of one or more degrees of freedom the molecule is bonded and restrained to an equilibrium position much as if it were in the solid state. Second, we recognize positional degeneracy due to the presence of vacant sites accessible

to a molecule at the price of storing up more or less elastic energy due to structural distortion as the molecule shifts to the new equilibrium position. Third, we must recognize that molecules which have escaped in one direction at least from fitting into the potential well made by neighbors may, in this liberated freedom, move much like a gaslike degree of freedom. Having recognized these significant structures, as was done in the iodine case, it still remains to estimate their concentrations.

X-rays indicate that the distance between near neighbors is closely the same for liquids as for solids, in spite of the over-all expansion. Thus it is natural to attribute the bonding energy to solid-like degrees of freedom and to attribute lack of bonding energy to gaslike degrees of freedom associated with localized loosening of the structure.

If we represent the energy of sublimation of the solid at the melting point by E_s, then the energy of vaporization of the liquid should be E_sV_s/V, where V_s is the volume of the solid at the melting point and V is the volume of the liquid. This follows because V_s/V is the average fraction of the space around a molecule filled by other molecules and is therefore the fractional degree of bonding of the liquid compared to the solid.

Just as in the iodine case the total bonding energy measures the fraction of the atoms which are present in molecular degrees of freedom, so in the liquid, we assume, the bonding energy measures the fraction, V_s/V, of solid-like degrees of freedom. The remaining fraction $(V\text{-}V_s)/V$ we take to be gaslike.

As a result of these considerations, we write for the partition function, f, of a normal liquid such as argon thus:

$$f = \left\{(1 - e^{-\theta/T})^{-3}e^{E_s/RT}(1 + n_he^{-a/n_hRT})\right\}^{NV_s/V}\left\{\frac{(2\pi mkT)^{3/2}}{h^3}eV/N\right\}\frac{N(V-V_s)}{V}. \tag{1}$$

Here n_h is the number of equilibrium sites accessible to a molecule in addition to its single most stable position; a/n_h is the strain energy stored in the system as the molecule shifts to one of the n_h sites. Undoubtedly, a/n_h should be written as $a/(n_h + \delta)$ since the strain energy when n_h is zero is very large but not infinite. However, to evaluate δ experimentally would involve a detailed knowledge of the glassy state not presently available.

The term eV/N in equation (1) comes from dividing the volume $(V - V_s)$, available to gaslike molecules, by the number of gaslike molecules, $N(V - V_s)/V$, and multiplying by the factor e, yielding the communal entropy associated with gaslike degrees of freedom. Such considerations involve making the assumption that gaslike degrees of freedom distribute themselves in such a way as to share communally the available volume, $V - V_s$. The factor eV/N in equation (1) likewise follows from the equality:

$$\left(\frac{(2\pi mkT)^{3/2}}{h^3}(V - V_s)\right)^{N(V-V_s)/V}(N(V-V_s)/V)!^{-1} = \left(\frac{(2\pi MkT)^{3/2}}{h^3}eV/N\right)^{N(V-V_s)/V}$$

θ, E_s, and V_s are the Einstein characteristic temperature, the energy of sublimation, and the volume of the solid, all measured at the melting point. We can regard these three quantities as parameters calculated from a quantum-mechanical treat-

ment of the solid state or as experimental solid-state parameters. The two new parameters characteristic of the liquid state are the extra sites per molecule, n_h, added in the liquid and the strain energy, a/n_h, required to occupy these sites. The pressure effect on viscosity would lead us to expect the formula

$$n_h = n(V - V_s)/V_s = 7(V - V_s)/V_s \quad (2)$$

for the number of holes, whereas

$$n_h = 10(V - V_s)/V_s \quad (3)$$

gives a better account of thermodynamic properties. Equations (2) and (3) would arise if seven and ten new equilibrium positions, respectively, arose each time the liquid volume, V, was increased by an amount V_s. A consideration of Figure 1 will convince one that these numbers are about right.

For the energy of distortion we require

$$a/n_h = 0.00285\ E_s V_s/(V - V_s), \quad (4)$$

where again it can be said that the linear dependence of strain energy on the inverse of the concentration of holes is a reasonable, if oversimplified, relationship, as is the magnitude of the proportionality factor 0.00285.

Returning to equation (1), one justifies the assumption that the extra volume $V - V_s$ is available to gaslike motions in spite of the degeneracy of solid-like degrees of freedom because all structures exclude the same volume, V_s. The strain energy, a/n_h, becomes negligible in its effect as volume and temperature increase. Thus in the neighborhood of the critical point, the term $n_h \exp(-a/n_h RT)$ reduces to n_h.

The partition function (1), with the values for the parameters n_h and a given by equations (3) and (4), permits the calculation of all thermodynamic properties for a normal liquid. The solid parameters used for the rare gases are listed in Table 1.

Some properties calculated for argon are tabulated in Tables 2–4.

TABLE 1

SOLID PARAMETERS AT MELTING POINT

	Neon	Argon	Krypton	Xenon
Energy of sublimation, E_s (cal.)	447.4	1,888.6	2,710.4	3,897.7
Solid volume, V_s (cc.)	13.98	24.98	28.4	36.5
Einstein characteristic temperature	44.7	60.0	45.0	39.2

TABLE 2

MELTING-POINT DATA FOR LIQUID ARGON

	Calculated	Observed
Volume (cc.)	28.00	28.03
Vapor pressure (atm.)	0.662	0.674
Temperature (° K.)	(83.85)	83.85
Melting entropy (e.u.)	3.66	3.35

TABLE 3

BOILING-POINT DATA FOR ARGON

	Calculated	Observed
Liquid volume (cc.)	28.48	28.69
Pressure (atm.)	1.05	1.00
Temperature (° K.)	(87.44)	87.44
Vaporization entropy (e.u.)	19.33	17.85

In Tables 2 and 3 the properties were calculated for the observed melting and boiling temperatures rather than for atmospheric pressure, which would have been only somewhat more laborious. To find the phases in equilibrium with each other, we plot $A = -kT \ln f$ for any desired T as a function of V and find the multiple tangent. The volumes at the various points of tangency are the volumes of the corresponding phases at the pressure given by the slope of the tangent. The tangent intercepts the axis A at the common Gibbs free energy for the various phases. Except at the triple point, a tangent line has, at most, two points of tangency. Tangents which cut the curve, A versus V, at any point correspond to a metastable state at the point of tangency which is not realizable as an equilibrium state but may be realizable kinetically.

TABLE 4

CRITICAL-POINT DATA OF ARGON

	CALCULATED		CALCULATED	OBSERVED
	$n = 6$	$n = 10$	(WALTER)	
Volume (cc.)	77.7	81.0	78.7	75.26
Temperature (° K.)	154.4	150.5	154.2	150.66
Pressure (atm.)	58.7	58.1	59.4	48.0

In Table 4 the values $n = 6$ and $n = 10$ refer to the respective values chosen for n in equation (2). The calculated critical properties depend only on the single liquid parameter n, since the distortional energy has become negligible at these volumes and temperatures. Calculated values in this and subsequent tables under the heading "Walter" come from one of two publications with that author and A. E. Stearn.[3,4] The Walter-Eyring partition function is conceptually similar to the partition function developed here and has been very successfully used in calculating the thermodynamic properties of liquids. The present development, however, represents a definite formal and conceptual simplification and improvement.

In Tables 5, 6, and 7 our calculated critical properties for neon, argon, krypton, and xenon are compared with Walter, Stearns, and Eyring's values and with the observed values. In these cases n in equation (2) was taken equal to 6, and the solid parameters used are given in Table 1. The slight changes obtained when $n = 10$ did not seem of sufficient interest to calculate at this time.

TABLE 5

CRITICAL TEMPERATURES (°K)

	$n = 6$	(Walter)	Observed
Neon	55.41	48.0	44.47
Argon	154.44	154.2	150.66
Krypton	208.33	215.0	210.6
Xenon	287.8	303	289.8

TABLE 6

CRITICAL PRESSURES (atm.)

	$n = 6$	(Walter)	Observed
Neon	37.65	32.7	26.86
Argon	58.72	59.4	48.00
Krypton	69.68	69.1	54.24
Xenon	74.89	75.6	58.2

TABLE 7

CRITICAL VOLUMES (cc.)

	$n = 6$	(Walter)	Observed
Neon	43.48	49.0	41.74
Argon	77.69	78.7	75.26
Krypton	88.32	81.0	...
Xenon	113.52	112.0	113.8

The critical properties above were calculated algebraically in the usual way, using

$$\left(\frac{\partial A}{\partial V}\right)_T = -p, \left(\frac{\partial p}{\partial V}\right)_T = 0, \left(\frac{\partial^2 p}{\partial V^2}\right)_T = 0.$$

The method of significant structures thus leads to a simple and surprisingly successful reduced equation of state for normal liquids. The deviations from experiment, particularly in the pressure, are such as would arise from insufficient account of clusters of molecules in the saturated vapor. Such clusters are known to exist. Extension of our partition function to abnormal liquids and to metals and molten salts are in progress along lines that have previously been found successful, using the Walter-Eyring partition function.

The authors wish to express appreciation to the National Science Foundation and one of them (N. H.) to the Research Corporation for their generous support.

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[2] Ralph Livingston, private communication.

[3] J. Walter and H. Eyring, *J. Chem. Phys.*, **9**, 393, 1941.

[4] H. Eyring, J. Walter, and A. E. Stearn, in *Surface Chemistry*, (Washington, D. C.: American Association for the Advancement of Science, 1943), No. 21, p. 88.

THE MODE OF PROTONATION OF AMIDES

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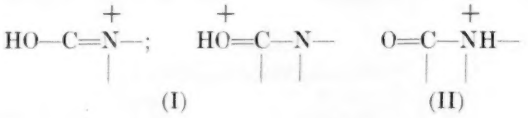
Communicated May 16, 1958

The relative basicities of oxygen versus nitrogen in amides is a problem which has not been satisfactorily resolved. However, it might be anticipated from considerations of resonance and structural parameters that in strongly acidic solutions the proton will attach to oxygen rather than to nitrogen, to give I rather than II:



(I) (II)

It is generally recognized that amides are monoprotonated in 100 per cent sulfuric acid. For example, O'Brien and Niemann[1] reported *i*-factors of 2.0, 2.7, 2.0,

TABLE 1

CHEMICAL SHIFTS OF AMIDES IN VARIOUS MEDIA*

$RCONR_1R_2$	SOLVENT	SHIFT				SPLITTING
		Solvent	RCO	NR_1R_2	$\Delta\nu$†	NR_1R_2
$HCON(CH_3)_2$	Pure liquid	...	−117.7	85.9‡	203.6	6.9
				92.0		
	H_2O	2.4	−125.9	75.1‡	201.1	6.7
				81.8		
	12 *M* HCl	−122.1	−143.9	59.5‡	203.4	5.9
				65.4		
	48% HBr	−95.7	−154.6	48.2‡	202.7	5.7
				53.9		
	98% H_2SO_4	−271.3	−141.1	51.0‡	192.1	4.7
				55.7		
	100% D_2SO_4	−280.5§	−144.9	49.4‡	194.3	4.6
				54.0		
$CH_3CON(CH_3)_2$	H_2O	5.9	106.8	68.0‡	38.7	6.0
				74.0		
	D_2O	...	106.8	68.0‡	38.7	6.0
				74.0		
	4 *M* Na_2SO_4 in H_2O	−4.4	98.8	60.4‡	38.4	5.8
				66.2		
	10 *M* $HClO_4$	−85.1	90.6	58.2	33.3	..
	5 *M* $HClO_4$	−24.3	98.8	64.2	34.6	..
	3 *M* $HClO_4$	−15.3	101.8	67.4	34.4	..
	1 *M* $HClO_4$	−3.6	105.4	69.3	36.1	..
$(CH_3)_2CHCON(CH_3)_2$	Pure liquid	..	113.9‡, ‖	76.1‡	..	8.1
			120.9	84.2	..	..
			152.7‡, ♯	..	..	..
			158.8	..	..	..
	100% H_2SO_4	−261.6	137.6‡, ♯	53.0‡	..	3.0
			124.9	56.0	..	
	100% D_2SO_4	−281.5§	135.3‡, ♯	49.0‡	..	2.7
			128.9	51.7	..	
$C_2H_5CON(CH_3)_2$	Pure liquid	...	162.5‡, **	65.3‡	...	10.6
			169.1	75.9	..	..
			109.4††, ‖	..	..	..
			115.7	..	..	..
			122.1	..	..	..
			127.6	..	..	..
			134.1	..	..	..
			140.6	..	..	..
	100% H_2SO_4	−267.5	134.0‡, **	45.0‡	..	7.3
			128.8	52.3		
$CH_3CONHCH_3$	Pure liquid	...	124.7	93.5‡	−118.3‡‡	4.0
				97.5		
	H_2O	1.8	113.4	82.5‡	..	3.8
				86.3	..	
	D_2O	3.6§	113.3	83.4	..	
	0.1 *M* HCl	...	95.4	69.4	..	
	0.1 *M* NaOH	...	111.0	81.9	..	
	60% $HClO_4$	−85.1	96.5	71.4	..	
	1% $HClO_4$	−5.2	112.1	82.1	..	
	100% H_2SO_4	−271.9	87.7	69.7‡	−144.8‡‡	4.2
				65.5	...	
	100% D_2SO_4	−286.5§	84.7	55.9	...	
CH_3CONH_2	H_2O	5.0	112.8	..	−98.0‡‡	
	100% H_2SO_4	−289.7	87.4	..	−147.9‡‡	

* In cycles from H_2O at 40 megacycles.
† Intramolecular chemical shift between N-methyl hydrogens and hydrogens of R group except where otherwise noted.
‡ Doublet.
§ Hydrogen of exchange and/or impurity.
‖ Methine hydrogen.
♯ Methyl hydrogens.
** Methylene hydrogens.
†† Sextuplet.
‡‡ N-H chemical shift.

2.9, and 1.9 for benzamide, glycinamide, trichloroacetamide, benzoylglycinamide, and phthalimide, respectively, in this solvent. Similarly, in this study we have found the *i*-factors of N,N-dimethylformamide, N,N-dimethylacetamide, and acetamide to be 2.1, 2.1, and 2.0, respectively. Thus the problem is to locate the locus of protonation in singly protonated primary, secondary, and tertiary amides.

Tertiary Amides.—It was thought that one might determine the site of protonation of a representative tertiary amide by examining the nuclear magnetic resonance (n-m-r) spectra of N,N-dimethylformamide in strongly acidic solutions. The pure liquid compound, as well as solutions of it in carbon tetrachloride and in water, has an n-m-r spectrum of three lines, one for the formyl hydrogen and a doublet for the methyl hydrogens. This splitting has been traced to a chemical shift between the methyl groups which results from hindered rotation about the carbon-nitrogen bond considered in I and II.[2] In strong acids, if the proton attached to oxygen one might expect retention of the methyl doublet since the carbon-nitrogen bond would still have considerable double-bond character as indicated in I. On the other hand, protonation on nitrogen should yield free rotation about the carbon-nitrogen bond and therefore collapse of the doublet. In fact, the methyl splitting of N,N-dimethylformamide was preserved in a variety of strong acids, e.g., 12 *M* HCl, 48 per cent HBr, 0.1–10 *M* $HClO_4$, including 100 per cent H_2SO_4 and D_2SO_4 (cf. Table 1).[3]

It might be argued, in the case of the hydrogen acids, that N-protonation had occurred, the N-H proton splitting the methyl hydrogens by spin-coupling. Deuterium couples very weakly,[4] especially through nitrogen.[5] Accordingly, the n-m-r spectrum of N,N-dimethylformamide was observed in 100 per cent D_2SO_4. The magnitude of the methyl splitting in this as well as in all other media tried was relatively constant throughout. Whereas the absolute positions of the peaks varied from one solvent to the next, the difference between the chemical shift of formyl hydrogen and the methyl hydrogens was substantially constant, indicating that the methyl hydrogens and the formyl hydrogen were equidistant from the center of charge on the molecule. The behavior of N,N-dimethylcyclopropylcarboxamide and N,N-dimethylisobutyramide also fell into this pattern. The methyl doublet of N,N-dimethylacetamide collapses reversibly in acidic solutions, from 0.1 *M* $HClO_4$ upward (cf. Table 1).

Secondary Amides.—The n-m-r absorption spectrum of N-methylacetamide in the liquid state, in nonpolar media, and in water at pH 7 consists of three peaks, a singlet for acetyl methyl and a doublet for the N-methyl hydrogens, which collapses to a singlet in the presence of most acids and bases and in D_2O. The effect of the first two types of solvents has been traced to a rapid exchange between N-H and solvent, i.e., the relaxation time for the N-methyl proton spins must exceed the half-life of N-H exchange.[5, 6] Since deuterium spin-couples but weakly,[4] N-deutero-N-methylacetamide would be expected to yield a single peak for the N-methyl hydrogens. The N-H peak itself is weak and quadrupole-broadened and is observable only in the pure compound.

In 96–100 per cent H_2SO_4 the methyl doublet returns, as does the N-H absorption. The doublet peaks are of equal amplitude with about the same separation as in the pure liquid. In D_2SO_4 the N-methyl doublet again collapses.

The above results again are consistent with O-protonation. There can be only

one proton on nitrogen, the other must be on oxygen. Acetylglycine shows the same behavior. It can also be concluded that the bisulfate ion is a poor base for exchanging the N-H proton of secondary amides in sulfuric acid.

Primary Amides.—The N-H proton magnetic resonance absorption of acetamide has been observed in saturated aqueous solutions at pH 7 and in 100 per cent sulfuric acid. Here, also, exchange of the N-H proton in sulfuric acid appears to be slow in comparison with its relaxation time. By measuring the areas under the absorption peaks it has been possible tentatively to assign O-protonation of acetamide in 100 per cent sulfuric acid. This interpretation is in accord with that of Hantzch, based upon ultraviolet spectroscopy,[7] and that of Huisgen and Brade,[8] based upon potentiometric pK determinations.

Experimental.—All spectra were determined with the Varian 4300 B, 40-mc., 9,400-gauss n-m-r spectrometer, equipped with spinner and superstabilizer. Samples were sealed in 5-mm. o.d. Pyrex tubes. Chemical shifts were calibrated by a combination of the method of side bands[9] and the use of toluene markers in capillary tubes as secondary standards. The cryoscopic measurements in 100 per cent sulfuric acid were conducted as described by O'Brien and Niemann.[1] The N,N-dimethylcyclopropylcarboxamide was kindly supplied by Mr. David Schuster. All other materials were obtained by the purification of samples of commercial origin.

The authors are indebted to Professor J. D. Roberts for his assistance during the course of this investigation.

* Contribution No. 2337.

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*OCCURRENCE OF CHROMOSOMAL ABERRATIONS IN PRE-SPERMATOCYTIC CELLS OF IRRADIATED MALE MICE**

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Communicated by C. C. Little, May 23, 1958

In both insects and mammals, only sperm which are mature at the time of irradiation are considered to form F_1 individuals, which bear major chromosome aberrations and particularly translocations. In the male mouse there is an initial period of approximately 1 month after the irradiation during which fertility remains good; offspring sired during this period bear appreciable numbers of genetic effects,

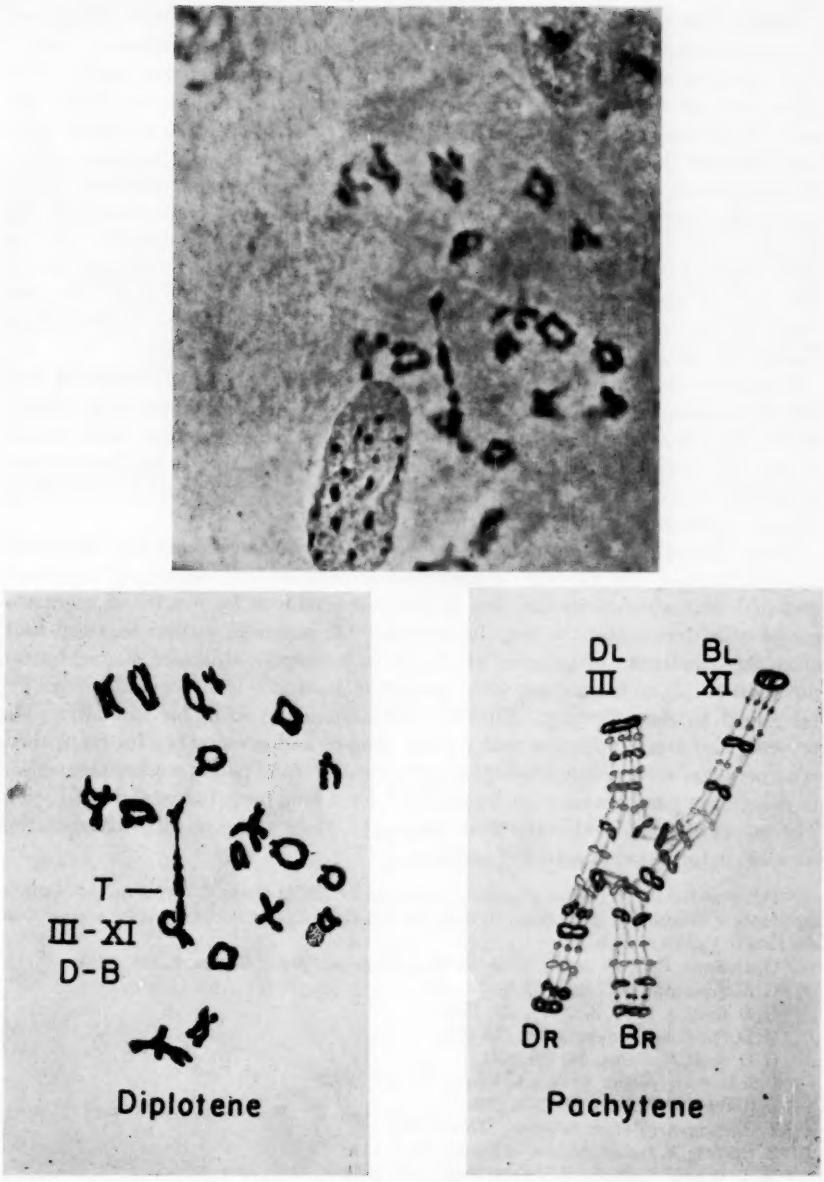
including mutations as well as gross chromosomal changes. Snell[1–5] and Hertwig[6–9] have shown that the fertile period is followed by one of sterility, the duration of which varies with the X-ray dosage; spermatogenesis ceases, and spermatogonia are few in number. A third period begins 6–8 weeks after irradiation, characterized by the reappearance of spermatogonia (presumably from primordia in the lining of the tubules) and of normal spermatogenesis; mature sperm appear, and fertility approaches normal. Snell, Hertwig, and Russell[10, 11] have observed that offspring produced during this lasting period of renewed fertility seldom showed the semisterility which is the classic indication of the presence of translocations and that mutations were infrequent; accordingly, it seems to have become a practice of subsequent investigators to discard irradiated individuals after their initial fertile period, for the expense and space requirements of maintaining mice beyond their period of obvious usefulness are extremely great. There have been few data on offspring conceived after the sterility period of the irradiated male parent.

TABLE 1

DISTRIBUTION OF SEMISTERILE F_1 INDIVIDUALS THROUGH MAINTENANCE PERIOD OF IRRADIATED P_1 MALES

Irradiated P_1 Male	Months Kept after Irrad.	Total F_1 Tested	Semisterile F_1 and Date of Birth in Months after Irradiation of Parent										
			1	2	3	4	5	6	7	8	9	10	11
1	6	26	4					1					
2	9	40						1					
3	9	62		2			1						
5	9	43		1				1					
6	9	44	2		1	1	4	1		1			
8	9	62			1	1			2				
10	9	64		4		1		2					
16	8	31	3		2		5		3				
21	8	28						2					
22	9	24	1	3		1		2					
26	10	45	1	2	1		1						
27	10	40	3						1				
28	9	29				4		4					
30	10	28	3		1		1					1	
40	10	29			1			2					
41	10	25			3		1						
42	10	33	1			4			1				
45	11	53	3	1				1					
		706	21	13	10	12	13	17	7	1		1	

In a project currently pursued by the writer, one portion of the study has required the production of chromosome aberration which serve as cytological markers for chromosome identifications in germinal and tumor cells. Young males from a base stock carrying the dominant sex-linked gene BENT-TAIL were irradiated at a total dosage of 700 r. from a beryllium window tube operated at 90–100 KVP and with filtration provided by 1 mm. of aluminum and 0.25 mm. of copper. Only the region of the testes was treated, the rest of the body being shielded with 1 mm. of lead. Immediately after the irradiation, each male was mated to four or more females of one of several standard inbred lines, including BALB/c, C57BL/6, and BR/cd, all of which have high fecundity. All F_1 individuals produced during the first 2 months in these matings were tested for semisterility, the indicator for chromosomal aberrations capable of producing aneuploid gametes (Snell, Hertwig,



Translocation of chromosomes B and D (linkage groups III and XI) of the mouse. L and R indicate the left and right map ends of the chromosomes.

Koller,[12] Russell). Numerous translocations were detected and have been established in breeding stocks.

Most of the 180 irradiated animals were discarded during their period of post-irradiation sterility; but 20 of the animals were held for further exploratory study. Each of these individuals was maintained for 6–11 months after the time of irradiation, and all offspring were tested for semisterility. Two proved to be permanently sterile, while the remaining 18 produced a total of 706 offspring which lived through the testing period. Of the 95 F_1 individuals showing semisterility, 26 were born 6 months or more after the time of the parent's irradiation, clearly beyond the time when sperm which were mature at the time of irradiation might still remain in the vasa and epididymes of the parents; it is therefore concluded that the sperm bearing the aberrations were produced from cells which received the irradiation in spermatogonial and even in pre-spermatogonial stages. For convenience, the term "pre-spermatocytic stages" is suggested as an inclusive one. Table 1 summarizes the data obtained from the 18 test individuals.

In order to determine conclusively whether the semisterility in the late-born individuals is caused by major chromosomal aberrations, breeding F_2 males were sacrificed from a line whose semisterile parent was born 7 months after the P_1 irradiation. Not only was a translocation found to be present, but it has further been shown to involve linkage groups III and XI. Figure 1 illustrates the translocation in a photomicrograph and in two drawings.

These findings have considerable significance in problems concerning the irradiation of humans. On the basis of past experimentation with the mouse, it has been assumed that aberrations are rare in gametes produced by irradiated spermatogonial cells (but mutations may be present). It has been further assumed that, after the exhaustion of gametes irradiated in post-spermatogonial stages, human procreation can be carried out with reasonable assurance that aberrations will not be passed to the offspring. Russell[11] summarizes the situation carefully. The present pilot study indicates that for the mouse, and presumably for man, there is no period of safety after irradiation; translocations and perhaps other aberrations in dangerous numbers may be transmitted for a long period after the irradiation. The extent of this period in the mouse is sought, along with a possible extrapolation for man, in further studies now in progress.

* This investigation is part of a program supported by USPH Grant C-1912 from the National Institutes of Health, by grant Instr. 70 from the American Cancer Society, and by a grant from the Elsa U. Pardee Foundation.

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SPACES FOR WHICH THE RICCI SCALAR R IS EQUAL TO ZERO

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Communicated February 3, 1958

The following study was made with the thought that the results might be of use in dealing with the so-called time symmetric gravitational wave solutions of Einstein's field equations.[1] At the time of symmetry, $T = 0$, the space part of the metric is arbitrary except for one condition, its Ricci scalar R is equal to zero.

1. The paper deals with a 3-space with positive definite quadratic form in terms of an orthogonal family of hypersurfaces, that is

$$ds^2 = \sum_i g_{ii}(dx_i)^2. \tag{1}$$

For this quadratic form the components R_{hihi} of the Riemann tensor are given by[2]

$$R_{hihi} = \sqrt{g_{ii}}\sqrt{g_{hh}}\left[\frac{\partial}{\partial x_h}\left(\frac{1}{\sqrt{g_{hh}}}\frac{\partial\sqrt{g_{ii}}}{\partial x_h}\right) + \frac{\partial}{\partial x_i}\left(\frac{1}{\sqrt{g_{ii}}}\frac{\partial\sqrt{g_{hh}}}{\partial x_i}\right) + \frac{1}{g_{ll}}\frac{\partial\sqrt{g_{ii}}}{\partial x_l}\frac{\sqrt{g_{hh}}}{\partial x_l}\right],$$

where h, i, and l are different.

The components R_{ii} of the Ricci tensor are given by[3]

$$R_{ii} = \frac{1}{g_{hh}}R_{hihi} + \frac{1}{g_{ll}}R_{liil} = \sqrt{g_{ii}}\left\{\frac{1}{\sqrt{g_{hh}}}\left[\frac{\partial}{\partial x_h}\left(\frac{1}{\sqrt{g_{ii}}}\frac{\partial\sqrt{g_{ii}}}{\partial x_h}\right) + \frac{\partial}{\partial x_i}\left(\frac{1}{\sqrt{g_{hh}}}\frac{\partial\sqrt{g_{hh}}}{\partial x_i}\right) + \frac{1}{g_{ll}}\frac{\partial\sqrt{g_{ii}}}{\partial x_l}\frac{\partial\sqrt{g_{hh}}}{\partial x_l}\right] + \frac{1}{\sqrt{g}}\left[\frac{\partial}{\partial x_l}\left(\frac{1}{\sqrt{g_{ll}}}\frac{\partial\sqrt{g_{ii}}}{\partial x_l}\right) + \frac{\partial}{\partial x_i}\left(\frac{1}{\sqrt{g_{ii}}}\frac{\partial\sqrt{g_{ll}}}{\partial x_i}\right) + \frac{1}{g_{hh}}\frac{\partial\sqrt{g_{ii}}}{\partial x_h}\frac{\partial\sqrt{g_{ll}}}{\partial_{xh}}\right]\right\}. \tag{2}$$

For the quadratic form (1) the contravariant components g^{ii} are given by[4]

$$g^{ii} = \frac{1}{g_{ii}}. \tag{3}$$

From (2) and (3) the Ricci scalar R, defined by

$$R = \sum_i g^{ii}R_{ii} = \sum_i \frac{1}{g_{ii}}R_{ii},$$

has the expression

$$R = 2\left\{\frac{1}{\sqrt{g_{11}}\sqrt{g_{22}}}\left[\frac{\partial}{\partial x_2}\left(\frac{1}{\sqrt{g_{22}}}\frac{\partial\sqrt{g_{11}}}{\partial x_2}\right)+\frac{\partial}{\partial x_1}\left(\frac{1}{\sqrt{g_{11}}}\frac{\partial\sqrt{g_{22}}}{\partial x_1}\right)+\right.\right.$$
$$\left.\frac{1}{g_{33}}\frac{\partial\sqrt{g_{11}}}{\partial x_3}\frac{\partial\sqrt{g_{22}}}{\partial x_3}\right]+\frac{1}{\sqrt{g_{22}}\sqrt{g_{33}}}\left[\frac{1}{\partial x_2}\left(\frac{1}{\sqrt{g_{33}}}\frac{\partial\sqrt{g_{22}}}{\partial x_3}\right)+\frac{\partial}{\partial x_2}\left(\frac{1}{\sqrt{g_{22}}}\frac{\partial\sqrt{g_{33}}}{\partial x_2}\right)+\right.$$
$$\left.\frac{1}{g_{11}}\frac{\partial\sqrt{g_{22}}}{\partial x_1}\frac{\partial\sqrt{g_{33}}}{\partial x_1}\right]+\frac{1}{\sqrt{g_{33}}\sqrt{g_{11}}}\left[\frac{1}{\partial x_1}\left(\frac{1}{\sqrt{g_{11}}}\frac{\partial\sqrt{g_{33}}}{\partial x_1}\right)+\frac{\partial}{\partial x_3}\left(\frac{1}{\sqrt{g_{33}}}\frac{\partial\sqrt{g_{11}}}{\partial x_3}\right)+\right.$$
$$\left.\left.\frac{1}{g_{22}}\frac{\partial\sqrt{g_{33}}}{\partial x_2}\frac{\partial\sqrt{g_{11}}}{\partial x_2}\right]\right\}. \quad (4)$$

For $R = 0$ the right-hand member of equation (4) equated to zero may be given the form

$$\frac{1}{g_{11}}\left[\frac{1}{\sqrt{g_{22}}}\frac{\partial^2\sqrt{g_{22}}}{\partial {x_1}^2}+\frac{1}{\sqrt{g_{33}}}\frac{\partial^2\sqrt{g_{33}}}{\partial {x_1}^2}-\frac{\partial\log\sqrt{g_{11}}}{\partial x_1}\left(\frac{\partial\log\sqrt{g_{22}}}{\partial x_1}+\frac{\partial\log\sqrt{g_{33}}}{\partial x_1}\right)+\right.$$
$$\left.\frac{\partial\log\sqrt{g_{22}}}{\partial x_1}\frac{\partial\log\sqrt{g_{33}}}{\partial x_1}\right]+\frac{1}{g_{22}}\left[\frac{1}{\sqrt{g_{33}}}\frac{\partial\sqrt{g_{33}}}{\partial {x_2}^2}+\frac{1}{\sqrt{g_{11}}}\frac{\partial^2\sqrt{g_{11}}}{\partial {x_2}^2}-\frac{\partial\log\sqrt{g_{22}}}{\partial x_2}\times\right.$$
$$\left.\left(\frac{\partial\log\sqrt{g_{33}}}{\partial x_2}+\frac{\partial\log\sqrt{g_{11}}}{\partial x_2}\right)+\frac{\partial\log\sqrt{g_{33}}}{\partial x_2}\frac{\partial\log\sqrt{g_{11}}}{\partial x_2}\right]+\frac{1}{g_{33}}\left[\frac{1}{\sqrt{g_{11}}}\frac{\partial\sqrt{g_{11}}}{\partial {x_3}^2}+\right.$$
$$\frac{1}{\sqrt{g_{22}}}\frac{\partial^2\sqrt{g_{22}}}{\partial {x_3}^2}-\frac{\partial\log\sqrt{g_{33}}}{\partial x_3}\left(\frac{\partial\log\sqrt{g_{11}}}{\partial x_3}+\frac{\partial\log\sqrt{g_{22}}}{\partial x_3}\right)+$$
$$\left.\frac{\partial\log\sqrt{g_{11}}}{\partial x_3}\frac{\partial\log\sqrt{g_{22}}}{\partial x_3}\right]=0. \quad (5)$$

From the form of this equation it follows that the expressions in brackets are not changed when $\sqrt{g_{ii}}$ for $i = 1, 2, 3$ are replaced by $e_i\sqrt{g_{ii}}$, where the e's are arbitrary constants. This fact may be used with solutions of equations (5) obtained below

2. Take

$$\sqrt{g_{11}} = {x_1}^{a_1}{x_2}^{b_1}{x_3}^{c_1},\ \sqrt{g_{22}} = {x_2}^{a_2}{x_3}^{b_2}{x_1}^{c_2},\ \sqrt{g_{33}} = {x_3}^{a_3}{x_1}^{b_3}{x_2}^{c_3}, \quad (6)$$

where the a's, b's, and c's are constants. For these expressions equation (5) is

$$\frac{1}{g_{11}}\left[c_2(c_2-1)+b_3(b_3-1)-a_1(c_2+b_3)+c_2b_3\right]{x_1}^{-2}+\frac{1}{g_{22}}\left[c_3(c_3-1)+\right.$$
$$\left.b_1(b_1-1)-a_2(c_3+b_1)+c_3b_1\right]{x_2}^{-2}+\frac{1}{g_{33}}\left[c_1(c_1-1)+b_2(b_2-1)-\right.$$
$$\left.a_3(c_1+b_2)+c_1b_2\right]{x_3}^{-2}=0. \quad (7)$$

From the form of this equation it follows that for any set of values of the a's, b's, and c's for which the expressions in brackets in equation (7) are equal to zero, the expressions (6) for these values are a solution of equation (5).

Several solutions are given herewith.

For $a_i = c_i = 0$ $(i = 1, 2, 3)$ one has $b_i = 1$, for which, from (6),

$$\sqrt{g_{11}} = x_2, \sqrt{g_{22}} = x_3, \sqrt{g_{33}} = x_1. \tag{8}$$

For $a_i = 0$ $(i = 1, 2, 3)$, $c_2 = b_3$, $c_3 = b_1$, $c_1 = b_2$, one has $c_i = {}^2/_3$, $b_i = {}^2/_3$ $(i = 1, 2, 3)$, for which, from (6),

$$\sqrt{g_{11}} = (x_2x_3)^{2/3}, \sqrt{g_{22}} = (x_3x_1)^{2/3}, \sqrt{g_{33}} = (x_1\,x_2)^{2/3}. \tag{9}$$

For $b_i = c_i = a$ $(i = 1, 2, 3)$, where a is an arbitrary constant, one has $a_i = {}^{3a}/_2 - 1$, for which, from (6),

$$\sqrt{g_{11}} = x_1^{3a/2-1}\,x_2^a x_3^a, \sqrt{g_{22}} = x_2^{3a/2-1}x_3^a x_1^a, \sqrt{g_{33}} = x_3^{3a/2-1}x_1^a x_2^a. \tag{10}$$

There is another approach to the problem, when it is noted that, in accordance with (6), the multipliers of the brackets in equation (7) are, respectively,

$$x_1^{-2(a_1+1)}, x_2^{-2b_1}, x_3^{-2c_1},$$

$$x_1^{-2c_2}, x_2^{-2(a_2+1}, x_3^{-2b_2},$$

$$x_1^{-2b_3}, x_2^{-2c_3}, \quad x_3^{-2(a_3+1)},$$

Since equation (7) is not a condition upon the x_i, the a_i, b_i, and c_i must be such that the above quantities are the same. The necessary and sufficient conditions for this are

$$a_1 + 1 = c_2 = b_3, a_2 + 1 = b_1 = c_3, a_3 + 1 = c_1 = b_2. \tag{11}$$

When these expressions for the b's and c's in terms of the a's are substituted in the brackets in equation (7) and the sum of the brackets in equation (7) is equated to zero, the result is

$$(a_1 + 1)^2 + (a_2 + 1)^2 + (a_3 + 1)^2 = 0.$$

From this result and (11) it follows that

$$a_i + 1 = 0, b_i = 0, c_i = 0 \ (i = 1, 2, 3),$$

from which and (6) one has

$$\sqrt{g_{11}} = \frac{1}{x_1}, \sqrt{g_{22}} = \frac{1}{x_2}, \sqrt{g_{33}} = \frac{1}{x_3}. \tag{12}$$

Take next

$$\sqrt{g_{11}} = x_2^{a_1} + x_3^b, \sqrt{g_{22}} = x_1^{a_2}, \sqrt{g_{33}} = x_1^{a_3}, \tag{13}$$

where a_i $(i = 1, 2, 3)$ and b are constants. For these expressions equation (5) is

$$\frac{1}{g_{11}}(a_2^2 + a_3^2 + a_2a_3 - a_2 - a_3)x_1^{-2} + \frac{1}{g_{22}}\frac{1}{\sqrt{g_{11}}}a_1(a_1 - 1)x_2^{a_1-2} + \frac{1}{g_{33}}\frac{1}{\sqrt{g_{11}}}b(b-1)x_3^{b-2} = 0. \tag{14}$$

This equation is satisfied by $a_1 = 1$, $b = 1$, and

$$a_2^2 + a_3^2 + a_2a_3 - a_2 - a_3 = 0. \tag{15}$$

Hence

$$\sqrt{g_{11}} = x_2 + x_3, \sqrt{g_{22}} = x_1^{a_2}, \sqrt{g_{33}} = x_1^{a_3} \tag{16}$$

for any values of a_2 and a_3 satisfying equation (15).

Since a's and b's must be such that equation (14) does not impose a condition upon the x's, it is necessary and sufficient that equation (15) hold and $a_1(a_1 - 1) = 0$ and $b(b - 1) = 0$. Hence, in addition to the above case $a_1 = b = 1$, there are three cases

$$a_1 = b = 0, a_1 = 0, b = 1, a_1 = 1, b = 0,$$

with the respective expressions for $\sqrt{g_{11}}$ from (13), namely,

$$\sqrt{g_{11}} = 2; \sqrt{g_{11}} = 1 + x_2; \sqrt{g_{11}} = x_2 + 1,$$

and expressions for $\sqrt{g_{22}}$ and $\sqrt{g_{33}}$ from (13) for values of a_2 and a_3 satisfying equation (15).

[1] J. Weber and J. A. Wheeler, *Rev. Mod. Phys.*, **29**, 509, 1957.

[2] *Ibid.*, p. 32.

[3] L. P. Eisenhart, *Riemannian Geometry* (Princeton, N.J.: Princeton University Press, 1926, 1949), p. 44.

[4] *Ibid.*, p. 44.

ON THE OPERATIONAL CALCULUS WITH DIFFERENTIABLE FUNCTIONS

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Communicated by A. Adrian Albert, May 12, 1958

In the present note, we shall consider a category of topological algebras[1] which present themselves naturally in the study of the general concepts of "differentiable functions" and of "differentiable spaces," without imposing the usual restriction of local euclideanness. We investigate here the question as to when topological algebras have an operational calculus with n-continuously differentiable functions; and we find the category of topological algebras A which are locally convex with respect to the collection of its ideals $I \subset A$ such that A/I is a pure algebra of differential order $\leq n + 1$ as a largest solution to the question, in a sense made precise in the statement of the theorem below.

We shall denote by $C^n(R)$ the topological algebra of all real functions f on the real line R having continuous derivatives up to the order n included, this algebra being endowed with the topology of order n, that is, of the uniform convergence of $f, \ldots, f^{(n)}$ on every compact subset of R. We shall also denote by $P(R)$ the sub-algebra of all real polynomials on R.

A topological algebra A is said to have an operational calculus with $C^n(R)$ when A satisfies the Hausdorff separation axiom and there is a continuous mapping

$C^n(R) \times A \to A$ denoted by $(f, x) \to f(x)$ such that, whenever $f \in P(R)$, then (fx) has its purely algebraic value. Such a mapping is then unique, by the Weierstrass theorem. More generally, we shall say that A has a pre-operational calculus with $C^n(R)$, provided that the natural mapping $P(R) \times A \to A$ given by $(f, x) \to f(x)$ is continuous as soon as $P(R)$ is endowed with the topology of order n induced on it by $C^n(R)$.

By a "local algebra" we shall mean an algebra with a unique maximal ideal. We shall say that an algebra A is of "finite differential order" when A is a direct sum of a finite number of ideals $A_1, \ldots, A_s$, which are local algebras whose maximal ideals $M_1, \ldots, M_s$ satisfy $M_1^{n_1} = \ldots = M_s^{n_s} = 0$ for some integers $n_1, \ldots, n_s \geq 1$. Alternatively, it is equivalent to request that A should have a finite number of maximal ideals and a nilpotent radical.[2] Such a direct sum decomposition is unique, apart from the ordering. In case $n_1, \ldots, n_s$ are requested to be the least such integers, then $n = \sum n_i$ is called the differential order of A.

We shall say that an algebra A is "separated" if, for every $x \in A$, $x \neq 0$, there is a pre-norm of algebra π on A such that $\pi(x) \neq 0$; in other words, in case the natural topology of A defined by all pre-norms of algebra on A satisfies the Hausdorff separation axiom. The algebra A will be called "pure" if all its maximal ideals are of co-dimension one.

THEOREM. *Let $\mathcal{C}$ be a category[3] of pure separated algebras whose radicals are nilpotent and $n \geq 0$ an integer. In order that every topological algebra A which is locally convex under itself[4] with respect to $\mathcal{C}$ should have a pre-operational calculus with $C^n(R)$, it is necessary and sufficient that $\mathcal{C}$ be a subcategory of the category $\mathfrak{D}_{n+1}$ of all pure separated algebras of differential order $\leq n + 1$.*

$\mathfrak{D}_{n+1}$ is therefore the largest category of pure separated algebras with nilpotent radicals assuring a pre-operational calculus with $C^n(R)$ in the sense of the above statement. The requirement in the statement of the theorem that the algebras of $\mathcal{C}$ be separated is not essential, as we may replace $\mathcal{C}$ by the category of the separated algebras associated with the algebras of $\mathcal{C}$, without affecting the situation.

The proof of the theorem rests on the following result.

LEMMA. *Let A be a pure separated algebra of differential order $n + 1$ and $\pi(A)$ be the set of all pre-norms of algebra of A. If $p \in P(R)$ and $r \geq 0$, define*

$$||p||_r = \sup\{\pi[p(x)];\ \pi \in \pi(A),\ x \in A,\ \pi(x) \leq r\}.$$

Then

$$\sup\left\{\sum_{i=0}^{n} \frac{\lambda^i}{i!} |p^{(i)}(t)|;\ \lambda \geq 0,\ |t| + \lambda \leq r\right\}$$

$$\leq ||p||_r \leq \sum_{i=0}^{n} \frac{(2r)^i}{i!} \sup\{|p^{(i)}(t)|;\ |t| \leq r\}.$$

This lemma implies that the topology defined on $P(R)$ by the family of pre-norms of algebra $p \to ||p||_r$ $(r \geq 0)$ coincides with the one induced on $P(R)$ by the topology of order n on $C^n(R)$.

The above theorem may be extended to the operational calculus with functions of finitely or infinitely many variables.

* The work reported in this note was done while the author was a fellow of the John Simon Guggenheim Memorial Foundation on leave of absence from the University of Brazil, Rio de Janeiro. This work was sponsored in part by the Army Algebras Research Project No. 2 at the University of Chicago.

[1] All algebras considered here are supposed to be commutative, with unit, over the field of real numbers. A pre-norm of algebra on the algebra A is a real function π on A such that $\pi(x) \geq 0$, $\pi(x + y) \leq \pi(x) + \pi(y)$, $\pi(xy) \leq \pi(x)\pi(y)$, $\pi(\lambda x) = |\lambda|\ \pi(x)$, $\pi(I) = 1$ or 0. A topological algebra is assumed to have its topology defined by its continuous pre-norms of algebra.

[2] See N. Jacobson, *Structure of Rings* (Coll. Pub. *Am. Math. Soc.*, No. 37 [New York, 1956]).

[3] We assume that if U belongs to $\mathfrak{C}$ and U is isomorphic to V, then V belongs to $\mathfrak{C}$.

[4] See L. Nachbin, these PROCEEDINGS, **43**, 935–37, 1957. The algebras are considered as algebras of operators on themselves.

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*CYTOLOGICAL AND CYTOCHEMICAL CHANGES IN LIVERS OF WHITE MICE FOLLOWING INTRAPERITONEAL INJECTIONS OF DNA PREPARATIONS FROM BREAST CANCERS OF AGOUTI C3H MICE**

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Communicated by Franz Schrader, May 23, 1958

Introduction.—In 1957 J. Benoit, P. Leroy, R. Vendrely, and C. Vendrely[1,2] reported changes in the characteristics of ducks of one breed following injections of deoxyribosenucleic acid (DNA) from another breed of ducks and stated that the modifications continued to be recognized in the progeny of the injected ducks. In view of the important implications which such observations may have for the role of the DNA as a genetic factor, we have started similar experiments, utilizing mice.

Although our own investigations are by no means far enough advanced to permit any conclusions in regard to the transforming activity of DNA in mice, a finding made incidentally during the course of these studies seems of sufficient significance to be reported in this communication.

It was noted that livers of white mice (BALB/C and CF_1) displayed abnormal cytological and cytochemical alterations after repeated intraperitoneal injections of DNA prepared from breast cancers of agouti C_3H mice. The alterations were not observed in controls or in white mice of the same strains after injections with DNA prepared from spleens and livers of the same C_3H mice.

Material and Methods.—Inbred strains of BALB/C mice of white color and low breast cancer incidence (less than 10 per cent) and of C_3H mice of agouti color with high breast cancer incidence (95 per cent) were obtained from the Jackson Memorial Laboratories. The white CF_1 hybrid mice were obtained from the Carworth Farms. The BALB/C and the CF_1 mice were bred further in our own laboratories, while the breast-cancer-bearing C_3H mice were sacrificed soon after their arrival and used for the DNA preparations. Livers, spleens, and the breast cancers were removed immediately after killing of the mice, and the DNA was prepared from each tissue according to the instructions of Vendrely and Vendrely.[3] Groups of newborn and

older mice from the BALB/C and CF_1 strains were injected intraperitoneally at repeated weekly intervals with the DNA preparations of the C_3H mice.

This report will be concerned only with groups of BALB/C and CF_1 mice which received their first DNA injection at the age of at least 3 months. Mice of the same age and sex were divided into four groups; the first group received DNA prepared from the breast cancer; the second, DNA from spleens; and the third, DNA from the livers. All three DNA preparations came from the same C_3H mice. Each group received a weekly intraperitoneal injection of approximately 0.3 cc. Density Units (D.U.)[4] of DNA for 3–6 weeks. The mice of the fourth group were not injected but were kept as controls.

At certain intervals after the injections, mice of each group were killed, and the organs were immediately removed and fixed in Lavdowsky's fluid under standardized conditions. Whenever possible, liver sections from control, spleen DNA-injected, liver DNA-injected, and breast cancer DNA-injected mice were mounted on the same slides so that simultaneous staining was possible. The PAS reaction for polysaccharides and Feulgen microspectrophotometry for the DNA determinations in individual liver nuclei were done as previously described.[5,6]

Results.—When BALB/C and CF_1 mice at 3–6 months of age received three to four weekly intraperitoneal injections of approximately 0.3 cc. D.U. of DNA[4] originating from the tumors of the C_3H strain, foci of unusually large atypical cells containing large nuclei and nucleoli, often multiple, were found scattered with varying frequency throughout the liver. Some of these bizarre cells were similar to the cell type observed in hepatomas, with chromatin which was frequently clumped and increased in quantity. Although extensive search of the livers revealed some mitotic figures, they were not too frequent; however, the microscopic appearance of many cells suggested an endomitotic or amitotic process.

When the PAS reaction for polysaccharides[5] was carried out on slides on which sections of control liver, liver from mice injected with spleen DNA, liver from mice injected with liver DNA, and liver of the mice injected with the tumor DNA were mounted simultaneously, a striking difference in the glycogen content of the cytoplasm of the liver cells was noted. While the liver-cell cytoplasm of the controls and of the mice injected with liver DNA or spleen DNA showed an abundance of glycogen, the cytoplasm of the mice injected with the tumor DNA showed frequently a striking decrease and sometimes a complete absence of glycogen, particularly in the large, abnormal cell type. On the other hand, the nucleoli, which were PAS-negative in the liver cells of the controls and of the spleen and liver DNA-injected mice, were strongly PAS-positive in the livers of the tumor DNA-injected mice, especially, again, in the abnormal cell types. The PAS positivity of the large nucleoli could be used as a convenient marker to study the distribution of the abnormal cell types within the liver parenchyma. The PAS reaction, which showed varying degrees of intensity in the nucleoli, also permitted the recognition of different structures within the nucleoli, such as small globules and fiber-like elements which were either PAS-negative or PAS-positive.

When quantitative determinations of the DNA content in individual liver cells were carried out by Feulgen microspectrophotometry,[6] a marked increase and a larger scatter of the DNA values were found in the livers of mice injected with the DNA prepared from the breast cancers. The liver cells from mice injected with the

same or even larger doses of the DNA derived from the spleens or livers of the same C_3H mice had essentially the same DNA values as did the ones of the non-injected controls. The increase in the DNA of the liver cells of mice was noted following the injections with tumor DNA, irrespective of whether mice were used in which the liver cells were predominantly diploid, tetraploid, or where ploidy formation from the tetraploid state to higher classes seemed to take place. An example of the latter in a control liver of a 6-month-old BALB/C mouse is given in Figure 2.

In Figures 1 and 2, characteristic examples of the DNA data obtained in the livers by Feulgen microspectrophotometry are presented. It can be seen in Figure

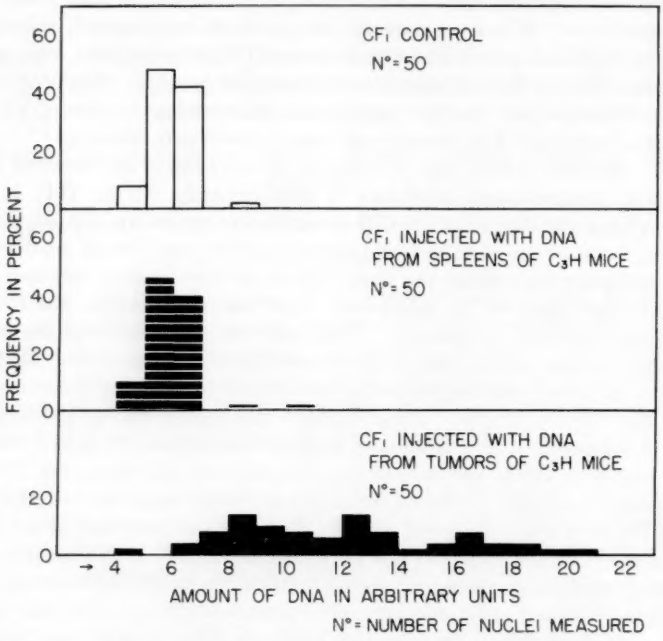

FIG. 1.—DNA content in individual liver nuclei of a control mouse, a mouse injected with spleen DNA, and a mouse injected with tumor DNA.

1 that the livers of the control and spleen DNA-injected CF_1 mice have not only the same mean tetraploid value of approximately 6 arbitrary units but that there are also a very similar distribution and a narrow range of the DNA values within the individual liver nuclei. This picture is very different from the DNA values in the liver of the mouse which has been injected with the tumor DNA; here no definite mean value is present, but, instead, a larger spread of DNA values above the tetraploid DNA can be noted. These DNA values are similar to those found in actively dividing and proliferating tissues, such as tumors.[7] Essentially the same findings are demonstrated in Figure 2, although, as mentioned above, the DNA val-

ues in the liver of the BALB/C control have a somewhat wider range, with a tendency toward a DNA peak near the octaploid value.

Discussion.—While it is not possible at the present time to interpret fully the various findings in the livers of BALB/C and CF_1 mice after injections with the DNA prepared from tumors of C_3H mice, nevertheless it is justifiable to state that the changes described point to a disturbance in the liver-cell metabolism. The occurrence of large, atypical cells with large nuclei and nucleoli and increased amounts of DNA strongly suggests a change in the nucleic acid metabolism, indicating nucleic acid synthesis and proliferation of the cells, although the latter appears somewhat atypical because of the scarcity of mitosis. The decrease in glycogen and the presence of PAS-positive nucleoli suggest an additional derangement in the carbohydrate metabolism. Whether there is an interrelationship between the nucleic acid and carbohydrate disturbances in these cells remains

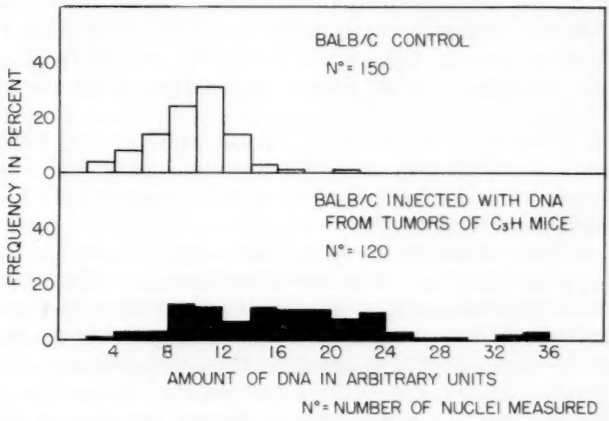

FIG. 2.—DNA content in individual liver nuclei of a control mouse and a mouse injected with tumor DNA.

to be seen, although there may be an inverse relationship similar to what has been observed in the salivary gland cells of *Helix pomatia* by Leuchtenberger and Schrader.[8]

The finding that the changes in the livers were noted, so far, only in mice which had been injected with tumor DNA, but not in those injected with DNA from spleen or liver derived from the same tumor-bearing mice, assumes special significance, since neither biochemical nor microspectrophotometric studies of the tumor, spleen, and liver DNA preparations revealed any significant differences between them. On the other hand, it is not too surprising that there exists a difference between the biological activity of the DNA derived from a tumor and of the DNA derived from a non-tumorous tissue. In recent years, evidence has been accumulated which indicates that the presence of a tumor in animals stimulates the nucleic acid and, in particular, the DNA metabolism of the normal organs of the host.[9]

C. Leuchtenberger, in 1954,[10] using Feulgen microspectrophotometry, also re-

ported an increase and wide scatter of DNA values in liver cells of mice bearing the rapidly growing subcutaneously transplanted Sarcoma 180. The similarity between these DNA data in the livers of tumor-bearing mice and the DNA data in liver cells of mice after injection with DNA prepared from tumors is indeed so striking that it may be suggested that the DNA contained in the tumor cells is perhaps responsible for the change of the nucleic acid metabolism in the normal tissues of tumor-bearing animals.

It is hoped that enzymatic studies which are under way will help to answer the question whether the biological activity is due to the tumor DNA itself, or whether the traces of proteins found in the DNA preparations (tumor, 0.8 μg. per D.U.; spleen, 0.3 μg. per D.U.; liver, 1.7 μg. per D.U.) may also be contributing factors. Regardless of the outcome of these studies, the fact remains that intraperitoneal injections of purified DNA preparations from tumor cells have a similar biological activity on normal tissues in vivo as has the presence of the tumor itself. While it is significant to find that the DNA from malignant tumor cells (breast cancer) displays a different biological activity from that of the DNA derived from non-tumorous tissues of the same animals, one is, of course, not justified to ascribe this activity to the malignancy per se. It may very well be possible that the activity of the tumor DNA is due to the fact that it is prepared from a rapidly dividing tissue. This is, however, not too likely a possibility, since the DNA from the spleen, which is also a dividing tissue, did not show such an effect on the liver. In order to explore this possibility further, we have prepared DNA from the embryonic tissue of the C_3H mice.

Another question, which also can be answered only in the future, concerns the fate of the atypical cells found in the liver after the tumor DNA injections. Will they progress to tumor formation (hepatomas), especially when the DNA injections are continued? Will perhaps partial resection leading to regeneration of liver tissue hasten the development of hepatomas? If the tumor DNA injections should ultimately produce tumors in normal tissues, then the concept that an aberrant DNA which may occur in cells due to virus infection, radiation, or other injuries may itself become a tumor-initiating factor will gain further support. Perhaps this approach may ultimately bring the long-sought answer to the question whether DNA itself can act directly as a tumor-promoting agent or whether it acts only in an indirect way as a mediator or cocarcinogen.

Summary.—Livers of white BALB/C and CF_1 mice show cytological and cytochemical alterations after repeated intraperitoneal injections of DNA prepared from breast cancers of agouti C_3H mice. The livers show foci of abnormally large cells containing large nuclei and large PAS-positive nucleoli. There is a decrease of the glycogen in the liver cytoplasm and an increase in the amounts of DNA in the liver nuclei.

These alterations were not present in livers of control mice or mice similarly injected with DNA derived from spleen or livers of the same C_3H mice.

The authors are greatly indebted to Drs. Colette and Roger Vendrely for making available to them a detailed description of the procedure for the preparation of the DNA. The authors also want to thank Mr. Allan Lerner and Miss Maria Maichle for their capable technical assistance.

* This investigation was supported by a research grant C-1814 from the National Institutes of Health, U.S. Public Health Service.

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*A MECHANISM OF CELLULAR THERMOGENESIS IN COLD-ADAPTATION**

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Communicated by H. W. Magoun, April 25, 1958

Introduction.—The adaptation of the non-hibernating, homeothermic mammal to lowered ambient temperatures could be achieved in theory either by reduction of heat losses or by an increase in energy exchange and heat production. It is by now well established[1–5] that, in general, the cold-adapted mammal, including man,[6,7] achieves this solution mainly by increasing its total metabolic energy production through higher caloric intake and increased oxygen utilization. Evidence from in vitro studies on slice[8–10] and liver brei[11] is also adequate to show that this response reflects adaptive changes at the tissue and cell levels, indicating use of preferred metabolic pathways of substrate utilization and energy exchange [12–14] by the cold-adapted system.

For optimal adaptation from the energetic standpoint, the required increase in heat evolution should be obtained by metabolic processes yielding the maximal heat compatible with the minimal requirements for basal maintenance work; whence the optimal system would appear to be one having, in terms of work, a relatively low efficiency, with a greater balance of its energy yield from substrate oxidation being diverted into a heat pool rather than into its usual form of potential energy in the chemical bond. With this, it would follow that, in the formation of ATP,[15] we should observe in the tissues of the cold-adapted rat a net decrease in phosphate fixation relative either to oxygen utilization or, implicitly, to total heat production. Under suitable experimental conditions this would appear as a decrease in the P/O ratio of the corresponding in vitro system.

The studies reported in this paper were undertaken primarily to test the foregoing proposition and, contingent upon the outcome, to examine either the metabolic pathways by which the expected result was achieved or, alternatively, the mechanism by which the cells of the cold-adapted system might achieve the necessary heat

evolution at minimal energetic cost. In this the underlying assumption has been made that the existence of such a mechanism would be highly probable in view of its obvious phylogenetic survival value.

The data presented here demonstrate that there is indeed a highly significant depression of the P/O ratio in liver mitochondria from the cold-adapted rat and that this is apparently not due to an increased degradation of ATP. Data are also presented on the extent of utilization by the cold-adapted animal of the poorly phosphorylating TPNH-cytochrome c pathway for terminal electron transport.[16] These results are discussed in terms of their possible relations to other pathways of an alternative nature and to cellular thermogenesis as an adaptive physiological response pattern of the cold-acclimatized mammal.

Methods and Materials.—Adult male rats of the Long-Evans strain were used exclusively. These were maintained in individual cages on water and a special ration[17] containing, by analysis, 25 per cent protein, 4–5 per cent ether-extractable components, and well supplemented with minerals and co-factors.

At initial weights of 180–200 gm., animals were placed in the acclimatization cold room (2° C. ± 1°) to be used only after 45–60 or more days of continuous exposure to the cold. Control individuals of the same age were maintained under identical caging and a maintenance temperature of 24°–27° C. All animals were routinely weighed weekly throughout and for a given set of experiments were paired off at sacrifice to match so far as practicable the sizes of the cold-adapted series to the same age batch.

Procedures of killing and preparation of tissue were most carefully controlled in respect both to time sequences and to the pre-killing treatment of the animals; in the latter, the killing was always done in the cold room of residence, and particular care was taken to avoid alarming the animals. Killing was by manual decapitation, using first a stunning blow with the back edge of a cleaver followed by a decapitating stroke, with a rapid excision of the liver within 30 seconds of the initial blow.

Mitochondria were prepared from liver tissue by methods modified after that of Schneider,[18] except that 9–12 gm. of excised liver were weighed, forced through a garlic press into three volumes of 0.25 *M* cold sucrose, and homogenized by 20 up-down strokes of a teflon pestle driven at about 750 rpm. The homogenate was centrifuged on the high-speed head of the International PR-1 at 0° C. at 950 $\times g$. for 10 minutes. The supernatant was then given 10 minutes at 25,000 $\times$ g. and the pellet re-suspended by syringe into 15 ml. 0.25 *M* sucrose. This fraction was used in all P/O measurements and as the mitochondrial fraction for the ATPase, transhydrogenase, and TPNH-cytochrome c reductase assays. The supernatant of the pellet was used for the assays of glucose-6-phosphate and 6-phospho-gluconic dehydrogenases.

Oxygen consumption was measured by the direct Warburg method and inorganic phosphate by the method of Gomori.[19] Nitrogen was determined by a modified Pregl micro-Kjeldahl method. Transhydrogenase assays were carried out by the method of Colowick *et al.*,[20] the TPNH-cytochrome c reductase by the technique of Horecker,[21] and the dehydrogenases of 6-phosphogluconate and glucose-6-phosphate by the methods of Glock and McLean,[22] using a Beckman DU spectrophotometer equipped with a recording attachment. The adenosine triphosphatase (ATPase) activity of mitochondria was measured as inorganic P released during a

20-minute incubation at 37° C. in tris-sucrose medium containing ATP and KF, each at 0.01 *M*.

Results.—Results (Table 1) on the oxygen utilization and P/O ratios of the in vitro mitochondrial systems show clearly that there was a marked and highly

TABLE 1

COMPARISON OF QO_2^N AND P/O RATIOS OF LIVER MITOCHONDRIA FROM NORMAL AND COLD-ADAPTED RATS

	Normal		Cold-adapted	
Medium	QO_2^N	P/O	QO_2^N	P/O†
A_2	103.2 ± 6.7‡	1.50 ± 0.108	73.6 ± 5.3	0.68 ± 0.055
B_2	93.5 ± 5.0	2.12 ± 0.096	75.5 ± 3.9	1.06 ± 0.092

* Mitochondria incubated at 37° C., hexokinase tipped after 5-minute equilibration. Oxygen consumption and phosphorus esterification measured for following 20 minutes. Medium A_2 contained (final concentrations) 0.25 *M* sucrose, 0.0015 *M* $MgCl_2$, 0.01 *M* Tris, 0.0003 *M* ATP, 0.008 *M* K phosphate buffer of pH 7.28, 0.008 *M* *dl*-β-hydroxybutyrate, 0.01 *M* KF, glucose and hexokinase. Medium B_2 contained 0.25 *M* sucrose, 0.006 *M* $MgCl_2$, 0.01 *M* Tris, 0.002 *M* ATP, 0.0001 DPN, 0.00015 *M* cytochrome c, 0.01 *M* *dl*-β-hydroxybutyrate, 0.01 *M* K phosphate buffer of pH 7.4, 0.01 *M* KF, glucose, and hexokinase.

‡ Standard error.

† *P* (normal-cold) < 0.001 in both media.

significant reduction in the P/O ratios of the particles from the cold-adapted animals, compared with their controls. One may note also that the addition to medium B of DPN and cytochrome c, while serving to raise the P/O values in both cold and normal, did not significantly alter the oxygen utilization. In each, the P/O ratios of the cold group were of the order of half those of the controls. It will be noted that the $QO_2{}^N$ values of the cold mitochondria in both media were significantly lower than those of the controls; it is believed that this may have been due to an impaired activation and oxidation of the ATP-dependent *d*(−) isomer of β-hydroxybutyrate occurring as a result of a reduced synthesis of ATP by the cold mitochondria.

Since the lowered P/O ratios of the cold particles could conceivably be caused by a greater fragility and consequent damage during the isolation procedures, measurements were made of the optical-density changes occurring on incubation of the mitochondria from the control and experimental animals. Figure 1 shows clearly that the normal and the cold mitochondria swell at the same rate when incubated in 0.25 *M* sucrose. However, when incubated in the medium used in the phosphorylation studies, the mitochondria from control rats swell somewhat faster than do those from cold-adapted animals. Since others have reported an increased rate of swelling to be associated with a lowered phosphorylative ability,[23] it would therefore appear that the lowered P/O ratios seen here in the cold-adapted animals are not due to an altered fragility of the mitochondria.

Whether the lowered P/O in the cold-adapted system is primarily due to a more active dephosphorylating mechanism or to a less efficient pathway of synthesis cannot be directly answered by the present experiments. However, measurements of degradation of ATP by mitochondria in substrate-free systems gave mean Q^N_{ATPase} (in μ atoms P/mg. N/hr) values which were identical (8.97 ± 0.54 and 8.96 ± 1.14) in the cold-adapted and control systems. A similar conclusion is confirmed in other studies, not detailed here, with liver homogenates derived from comparably adapted rats. Likewise, the addition of fluoride to such systems, while

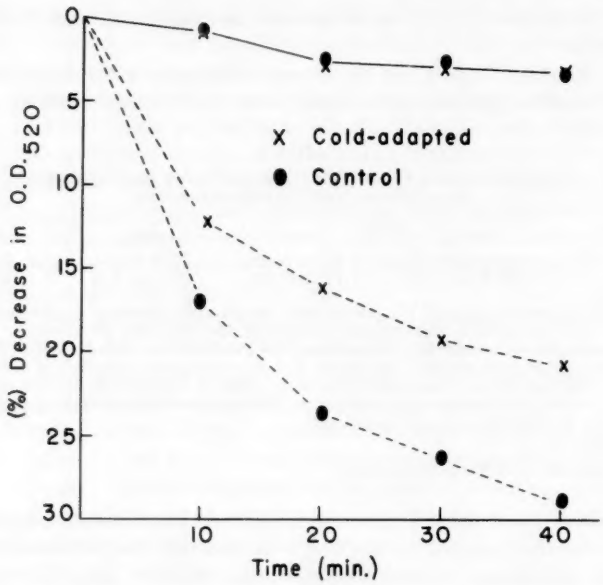

FIG. 1.—Optical-density changes in liver mitochondria of normal and cold-adapted rats incubated in vitro. Mitochondria were added to 0.25 *M* sucrose (———) or to the medium used in the phosphorylation experiments (– – – –), incubated at 26° C., and the optical density at 520 mμ measured. Values shown represent percentage changes from the initial optical density.

of course reducing the absolute levels of ATPase activity, has in no way altered the conclusion that no difference exists in the net degradation of ATP. The inference, then, is that there is a decreased synthesis of ATP in the cold-adapted mitochondria.

The essential basis for a deviation in metabolic pathway along the electron transfer chain is suggested from the works of several authors [22,24,25] who have variously shown that in experimental hyperthyroidism there is a facilitatory change in activities of a number of enzymes favoring enhanced TPNH formation and oxidation. To the extent, then, that in the cold-adapted animal there is increased thyroid activity,[26–29] an analogous situation could obtain.

Assays of some of the enzymes involved in TPNH formation and oxidation were therefore made, with the results as given in Table 2. From this it appears that the only difference of possible significance is that of the transhydrogenase ($P = 0.07$). Since this is in the right direction to favor the TPNH pathway, however, it may be suggested that some functional significance may attach thereto in achieving the observed depression in oxidative phosphorylation of the cold-adapted system.

It may be noted here that the enzyme activities as measured in vitro represent essentially maximal rates, since the substrate is in excess, while in vivo the same enzymes may not be functioning at maximal rates, depending on the substrate

concentration. Depending on the extent to which these enzymes are operating in the mitochondria of the respective systems, it might therefore be that only small changes in amounts of transhydrogenase and TPNH-cytochrome c reductase would be necessary in the cold-adapted animal to produce the observed alteration in P/O; it may be that such small changes in activities do occur but are not detected under our assay conditions.

Discussion.—From the present data it is not yet possible to conclude the extent to which the thermogenic potential of the cell in vivo is governed by the observed reaction system. Certainly, it can be stated only that some alteration in metabolic pathways has occurred in which a net thermogenic increment is made available to the cold-adapted system and that this does not appear in a comparable system from the unacclimatized control. For the substrate system as employed in vitro, the cold-adapted animal would appear to enjoy a caloric advantage of the order of a factor of 2 over the non-adapted state. Thus, for the same amounts of oxygen consumed and substrate oxidized, the resting heat production of the liver would, on this basis, be doubled in the acclimatized animal. Conversely, of course, the work potential of the system would be correspondingly reduced, whence the substrate requirements for a given maintenance level would be proportionately increased.

TABLE 2

COMPARISON OF SPECIFIC ACTIVITIES* OF ENZYMES FROM LIVERS OF NORMAL AND COLD-ADAPTED RATS

Enzyme	Normal	Cold-adapted
Transhydrogenase	0.140 ± 0.011†	0.094 ± 0.020‡
TPNH-cytochrome c reductase	3.701 ± 0.302	3.968 ± 0.368
Glucose-6-PO_4 dehydrogenase	0.723 ± 0.097	0.654 ± 0.051
6-Phosphogluconic dehydrogenase	1.851 ± 0.195	1.814 ± 0.196

* Activities are expressed in arbitrary units per milligram nitrogen.

‡ Standard error.

† $P = 0.07$.

Moreover, if these conclusions can be applied to the whole animal, it may be inferred that the caloric cost of a given work load will be higher in the cold-adapted animal than in the normal.

Concerning the biochemical mechanisms, it is not explicitly clear how the reduced efficiency of phosphorylation is achieved in the cold-adapted animal. From the ATPase measurements it would appear that the mitochondria from both cold and normal rats are equally capable of dephosphorylating ATP. Whence, if it can be assumed that exogenous ATP penetrates to the internal sites of normal biological exchange within the particles, then it follows that the low P/O ratios in mitochrondria from the cold-adapted rat reflect a lowered net ATP synthesis. The latter could be achieved if the cold-adapted animal utilized to a greater extent the route of terminal electron transport through the poorly phosphorylating TPNH-cytochrome c reductase pathway rather than that through the more efficient pathway involving DPNH. However, it would appear from our measurements of activities of the transhydrogenase and TPNH-cytochrome c reductase systems that this shunting does not occur to any significant extent. It is admittedly difficult to see why this electron transfer shunt through TPNH should not be utilized by the cold-adapted animal. However, the actual physiological significance of this pathway is itself

not altogether clear in respect to in vivo thyroid activity; the experimental observations which led to the postulate of the shunt in hyperthyroidism[24] were actually obtained with animals receiving thyroxin at levels of the order of 1,000 or more times greater than those obtaining in vivo in either normal or cold-adapted animals. On this account interpolation to physiological steady-state levels would appear of uncertain validity.

It appears entirely possible that other alternative pathways are utilized during cold-adaptation; one possibility is shown schematically in Figure 2. In this it is postulated that an intermediate $Z \simeq P$ is formed in the mitochondria of the cold-adapted rat; if this intermediate requires an appreciable energy input for synthesis and further has a very short half-life, then the inorganic phosphate pool of the experimental system would remain unchanged, irrespective of the turnover rate of the $Z \simeq P$, whence a lowered P/O would ensue. It is necessary further to postulate

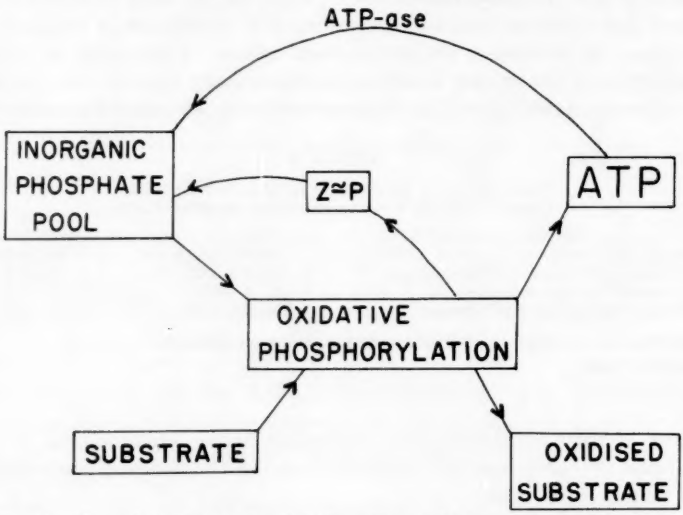

FIG. 2.—Suggested scheme of metabolic pathways in liver mitochondria of the cold-adapted rat. For explanation see text.

that the phosphate donor in the synthesis of $Z \simeq P$ is not ATP, since the ATPase assays would otherwise measure also the degradation of ATP by way of $Z \simeq P$. While these two routes of degradation are currently experimentally indistinguishable, they are in any case equal in the cold and normal mitochondria. The phosphate donor might then be a phosphorylated intermediate of the electron transport chain which reacts preferentially with Z to form $Z \simeq P$, rather than with the phosphate acceptor system which normally leads to ATP synthesis. While this scheme is admittedly hypothetical, experiments are now in progress to test certain of its implications.

Addenda.—(1) Since conclusion of these studies it has also been suggested by Potter[30] that a "calorigenic shunt" could occur in cold adaptation through preferential operation of the TPNH-cytochrome c pathway to give a net lowering of phosphorylation.

(2) Concurrently with our preliminary report,[10] Panagos and Beyer[31] reported that P/O ratios of liver mitochondria with succinate were depressed variously, depending on the extent of the cold acclimatization period. Since succinate is known to react at the flavin level of the electron transport system, these results may possibly indicate that a major difference in electron transport phosphorylation of the cold acclimatized liver mitochondria appear in the step, pyridine nucleotide to flavin.

* This work was supported largely by United States Public Health Service Research Grants C-3103 and C-3635, from the National Cancer Institute, National Institutes of Health.

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*EPIGENETIC CONTROL SYSTEMS**

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Communicated by T. M. Sonneborn, May 8, 1958

Advances in chemical genetics in the past few years have permitted the formulation of a consistent hypothesis concerning the chemical nature of the "primary" genetic material.[1] This material is considered to consist of nucleic acid (usually deoxyribosenucleic acid) and to contain genetic information in a code of prescribed nucleotide sequences. The information is believed to be preserved during replication by a "semiconservative" reproductive mechanism, whereby each daughter strand retains a half of the parental molecule and reconstructs a complementary portion. Alterations in the genetic material are thought to come about in one of two major fashions. Mutations are the more or less random alterations in the code which result from chance substitutions in the nucleotide sequences or from gains or losses of nucleotides. Recombination also results in changes in the code, but these changes are achieved in a more orderly fashion; they require the physical association of different kinds of genetic material and are limited in their possibilities in any given instance by the nucleotide sequences in the parental materials. According to this hypothesis, the genetic material expresses its specificity through a decoding process, by which the information in the nucleotide sequences is eventually translated into, for example, amino acid sequences in proteins.

This view of the nature of the genetic material, while certainly not established in detail, finds much support in experimental studies and gains great strength from its simplicity. It permits, moreover, a clearer conceptual distinction than has previously been possible between two types of cellular control systems. On the one hand, the maintenance of a "library of specificities," both expressed and unexpressed, is accomplished by a template replicating mechanism. On the other hand, auxiliary mechanisms with different principles of operation are involved in determining which specificities are to be expressed in any particular cell. Even without specifying precisely how these other mechanisms operate, the distinction between mechanisms involving template replication and "other mechanisms" is reasonably clear, even though both are involved in determining cellular characteristics. Difficulties arise, however, when one attempts to determine whether observed differences in cellular properties are due to differences in the "primary genetic material" or to differences in other cellular constituents. Some of these difficulties can be made apparent by setting forth certain general propositions related to the supplementary regulatory systems for which evidence is now available. To simplify the discussion of these two types of systems, they will be referred to as "genetic systems" and "epigenetic systems." The term "epigenetic" is chosen to emphasize the reliance of these systems on the genetic systems and to underscore their significance in developmental processes.[2]

1. *Cells with the Same Genetic Material May Manifest Different Phenotypes.*—Although this proposition cannot be directly demonstrated to be true, abundant circumstantial evidence for its validity is available. Certainly, microbial cells grown on different substrates or cells found in different tissues of a higher organism

can be distinguished on many bases—morphological, physiological, and biochemical. Yet the available evidence makes it unlikely that these cells have changed their genetic information in the process of becoming different. The existence of phenotypic differences between cells with the same genotype merely indicates that the expressed specificities are not determined entirely by the DNA present in the cell—that other devices, epigenetic systems, regulate the expression of the genetically determined potentialities.

2. *The Genetic Potentialities of a Cell Are Expressed in Integrated Patterns.*—The determination of expression or non-expression of a particular specificity is seldom, if ever, an independent determination for each specificity stored in the "genetic library." In many cases, as in the sequential induction of enzymes, the expression of one specificity may involve the eventual expression of a series of specificities. Conversely, the expression of one specificity may make impossible the expression of other potentialities. Mutual exclusion, in a gross sense, is apparent throughout embryonic development; a cell cannot function as a nerve cell and as a liver cell at the same time. Phase variation in *Salmonella*,[3] serotype variation,[4] and mating-type variation[5] in certain ciliates provide examples of mutual exclusion in micro-organisms. Both simultaneity of expression and mutual exclusion of expression imply that intercommunication and metabolic linkage are important characteristic features of epigenetic systems.

3. *Particular Patterns of Expression Can Be Specifically Induced.*—This is, of course, a basic generalization in developmental biology; cells develop in particular ways in part at least because they occupy certain geographical locations and are exposed to certain chemical environments. Similarly, directed changes in micro-organisms, within limits set by the genotype, can be observed; induced enzyme synthesis is a special case of this phenomenon. The specific agents of induction need not be extrinsic in origin, however, but may be produced within a single cell. This proposition of specificity of induction is a corollary of the previous proposition, since integration and specific inhibition would be difficult or impossible without intracellular communication by specific agents. Moreover, a large part of the utility of epigenetic systems for micro-organisms lies precisely in their ability to respond specifically to altered environmental conditions.

4. *Epigenetic Systems Show a Wide Range of Stability Characteristics.*—Certain patterns of expression, although specifically induced, may be perpetuated in the absence of the inducing conditions. For this reason, cells with the same genotype may not only manifest different phenotypes, but these differences in expressed potentialities may persist indefinitely during cellular division in essentially the same environment. The widely held view that differentiated cells in vertebrates do not "de-differentiate" suggests that some epigenetic systems have great stability; the lack of universal agreement on this view suggests that epigenetic systems vary in stability. The observation that cells of *Escherichia coli*, adapted and unadapted to galactoside, can be maintained indefinitely in the same environment provides a recent and striking example of a stable epigenetic homeostat.[6] Other examples in unicellular forms are, again, the systems regulating serotype and mating-type specificities in the ciliates.[4, 5]

These observations create a real problem. One operational definition for "hereditary differences" has involved the indefinite perpetuation of cellular dif-

ferences during growth in the same environment. Yet instances are known in which cellular differences may be maintained in the absence of detectable genetic or environmental differences. Hence the observation of indefinite persistence of differences does not distinguish persistent homeostasis due to DNA maintenance (genetic homeostasis) from persistent homeostasis due to epigenetic regulation (epigenetic homeostasis). Moreover, great difficulty is encountered in separating, on any conceptual basis, epigenetic systems controlling differences which persist indefinitely from systems maintaining differences for shorter periods of time, and these, in turn, from systems relating to differences which disappear immediately when cells are placed together in the same environment. "Cellular memory" is not an absolute attribute. Thus the permease system mentioned above[6] shows persistence during growth under one set of environmental conditions but not under another set of conditions. Moreover, what appear to be very similar systems of control of serotype specificities in varieties 1 and 4 of *Paramecium aurelia*[4] differ considerably in their ability to maintain differences in a common environment; even within a single variety wide variations in serotype stabilities are observed, depending upon which specificities are being expressed and what cultural conditions are employed. Therefore, the separation of epigenetic regulators into two major classes, depending on their stability, would separate regulators with fundamentally similar mechanisms into two artificial categories and might even place the same system in different categories, depending on the conditions of observation. This variability in stability of a single epigenetic system may be a very common phenomenon; the major operational difficulty lies, however, in separating genetic systems from epigenetic systems under conditions of maximal stability.

5. *Some Epigenetic Devices May Be Localized in the Nucleus.*—Some attempts to characterize cellular regulatory agencies have placed considerable reliance on a geographical distinction; the genetic systems were considered to reside in the nucleus and on the chromosomes, to be stable and insulated from environmental alterations. The supplementary systems were thought to occupy the cytoplasm, to be more flexible and responsive to environmental alterations. While this distinction may have some general validity, its usefulness in particular cases may be slight. First, some of the systems of greatest interest are not amenable to the operations permitting a distinction between nuclear and cytoplasmic bases, i.e., breeding analysis or nuclear transplantation. Second, some genetic material occurs in the cytoplasm, although its common occurrence there is debatable. More serious are observations which suggest that some epigenetic control systems are located in the nucleus. The studies on nuclear differentiation in amphibian development[7] provide perhaps the most dramatic single example of this evidence. Studies on the serotypes in *Salmonella*,[3] however, go even further in suggesting that such control systems may even be localized on the "chromosomes" themselves. If such systems are so localized and particularly if they manifest considerable stability, they would behave in breeding analyses in a manner strictly comparable to genetic systems and would be indistinguishable from them on this basis alone. Certainly, when breeding analysis demonstrates a chromosomal localization of a determinant of cellular differences, strong evidence must be available to counterbalance the presumption of genetic control, but in some instances chromosomal control systems manifest so many "typical" epigenetic characteristics that their classification as such may be warranted.

This discussion then leads directly to the critical question: Can any available operation, or combination of operations, yield an unambiguous classification of cellular regulatory systems into genetic and epigenetic categories? Individually, persistence and nuclear localization have been rejected as adequate criteria. While a lack of persistence excludes a genetic basis for differences, the observation of persistence does not exclude an epigenetic basis. Although indicative, neither cytoplasmic nor nuclear localization certainly classifies a control system, since some genetic elements exist in the cytoplasm and some epigenetic systems are found in the nucleus.

If these commonly used criteria are inadequate, what other criteria can be employed? One criterion of possible utility concerns the specificity of induction of changes, but this criterion is also subject to ambiguous interpretation. Epigenetic systems are useful biological systems primarily because of their susceptibility to control; integration without specific inhibition and specific induction would be impossible. In principle, therefore, epigenetic alterations should be predictable and, under the proper circumstances, should occur with great reliability. In practice, however, a failure to induce a change with predictable results might reflect nothing more than a lack of knowledge about the proper conditions. Moreover, certain types of genetic alterations can be achieved with precision. Thus cytoplasmic genetic elements may be lost by specific environmental manipulations, and specific introduction of genetic material (as in virus infection) may not be detected as such, yet may lead to specific genetic alterations. Moreover, quantitative changes in the genetic material may be achieved by known treatments, and differential quantitative changes may occur within a single cell. One cannot even entirely exclude the possibility of specific mutagens, in the sense of agents which reliably alter particular nucleotide sequences in a particular manner, though convincing evidence for such agents is not yet available. In spite of these difficulties, one might expect epigenetic systems to be less stable and more susceptible to extrinsic control than genetic systems, and this criterion may be of some value.

Finally, the restrictions imposed on epigenetic systems by the genetic material require that the alterations which occur be limited by the information available in the genetic library. Hence one may in certain cases find suggestive evidence for epigenetic control if the number of "states" of a system is limited.

These criteria must be recognized as individually inadequate, unsatisfactory in combination, and provisional at best. Their application at the present time must involve a considerable subjective factor. Nevertheless, they constitute our chief barricade from nihilism and provide a basis for tentatively classifying several chromosomal homeostats as epigenetic.

A detailed analysis of all the systems which show presumptive epigenetic characteristics, or even those which may involve nuclear epigenetic homeostats, is not possible in this essay. Nevertheless, a few such systems and their more significant attributes will be mentioned briefly. In certain cases, where breeding analyses of somatic nuclei are not possible, a nuclear localization of control systems has been established by other means. These cases are considered to involve epigenetic control for the following reasons. The persistent nuclear alterations studied by Briggs and King,[7] in so far as they can be reckoned to be normal alterations occurring during development, were probably induced by specific, but unknown, local conditions of the embryonic environment. The nuclear alterations responsible for

mating-type differentiation in the ciliates[5] may also be specifically induced, in some cases by the cytoplasmic environment; they show, moreover, a limitation of the spectrum of changes which is directly imposed by the genotype.

The system of phase variation in *Salmonella*, studied by Lederberg and Iino,[3] has been analyzed by transductional techniques and shown to be localized on the "chromosome." The epigenetic character of the system is suggested by the relative instability of the homeostat but is indicated much more strongly by the limitation of the spectrum of changes to stable specificities controlled by two genetic loci. Some of the changes in this system have not yet been brought under direct control, but other changes appear to be directed by internal regulatory agents.

The studies of Brink and his co-workers[8] on modifications at the R locus in maize present another situation of considerable interest. Certain chromosomally localized alterations are specifically induced when particular homologous genetic elements are brought together in heterozygotes; these modifications may persist indefinitely after the inducing conditions are removed but can be reversed to a certain extent under other specific intracellular conditions. This study bears many resemblances to that of Rizet[9] on the "barrage" effect in *Podospora*, who also demonstrated a specific cellular alteration, induced in a particular heterozygote, associated with a particular genetic locus, and persistent after the heterozygous condition was resolved. Moreover, a reversal of the alteration could be achieved by exposure of the modified strain to cytoplasm of an unaltered strain. Although some of these changes in both maize and *Podospora* occurred in known heterozygotes, recombination is considered an unlikely explanation, since no detectable alterations occurred in one of the allelic loci and since the cells receiving the other locus were modified in 100 per cent of the cases. (Other alterations occurring specifically in heterozygotes are difficult to distinguish from recombination of some sort, particularly when they occur only very rarely; the possibility, however, that some rare interallelic effects—such as in "gene conversion"—are due to epigenetic alterations cannot be discounted.)

Finally, the studies of Smith and Sand[10] suggest that epigenetic systems may be responsible for some of the phenomena ascribed to "mutable genes." They extracted an unstable locus from an interspecific hybrid of *Nicotiana* which shifted frequently between two forms in both somatic and germinal tissue. A specific induction of the alterations is suggested by the fact that the alterations in the soma appear to occur at specified times in development, different for the two directions of change. The rates of change are, moreover, highly and differentially sensitive to temperature. The detection of this unstable gene in an interspecific hybrid suggests that the gene, removed from the genome of origin, is incapable of responding in a regular fashion to normal development stimuli in a foreign genome.

This discussion is based on the idea of two types of cellular regulatory systems, both capable of maintaining persistent cellular characteristics but achieving homeostasis by different means. The current concept of a primary genetic material (DNA), replicating by a template mechanism, is opposed to a homeostatic system operating by, perhaps, self-regulating metabolic patterns.[11] The details of operation in neither type of system can be precisely determined at the present time, and any attempt to discriminate between them may be premature. Certainly, an operational distinction at the present time encounters great difficulties. Never-

theless, a recognition of the existence of the two types of systems, and even the difficulties in distinguishing between them, may be useful in avoiding confusion in discussing cytoplasmic inheritance, developmental alterations, inheritance of acquired characters, mutation, and genetic recombination.

* Aided by grants from the National Science Foundation. This essay is based on remarks given at a conference on "Extrachromosomal Heredity" held at Gif-sur-Yvette, France, in March, 1958. The conference was held under the auspices of the Rockefeller Foundation and the American Institute of Biological Sciences.

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ERRATA: STIMULATION OR INHIBITION OF VIRUS OF INFECTED AND INSECT-GALL TISSUES AND SINGLE-CELL CLONES

BY A. C. HILDEBRANDT

The title of the article appearing in these PROCEEDINGS, **44,** p. 354, should read Stimulation or Inhibition of Virus Infected and Insect-Gall Tissues and Single-Cell Clones

ERRATA: NOMENCLATURE OF THE ACTINOMYCINS

BY SELMAN A. WAKSMAN, EDWARD KATZ, AND LEO C. VINING

In the article of the foregoing title appearing in these PROCEEDINGS, **44,** p. 608 should appear as follows:

TABLE 4

INFLUENCE OF NITROGEN SOURCE ON COMPOSITION OF ACTINOMYCIN COMPLEX

ORGANISM	NITROGEN SOURCE	RELATIVE PERCENTAGE OF COMPONENTS						
		I	II	III	IV	V	VI	VII
S. antibioticus	L-glutamic acid	6.2	2.1	2.8	80.4	8.4	..	..
S. antibioticus	L-threonine	7.1	2.4	2.9	29.2	58.4	..	..
S. antibioticus	L-glutamic acid plus sarcosine	7.1	24.6	35.6	27.2	5.5	..	..
S. chrysomallus	KNO_3	..	..	..	10.0	..	50.0	40.8
S. chrysomallus	Glycine	..	..	..	7.0	..	43.2	49.8

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Schwarzschild, Martin, 1956 (2), Princeton University Observatory, 14 Prospect Avenue, Princeton, New Jersey

Schwinger, Julian, 1949 (3), Department of Physics, Harvard University, Cambridge 38, Massachusetts

Seaborg, Glenn Theodore, 1948 (5), Department of Chemistry, University of California, Berkeley 4, California

Seares, Frederick **H**anley, 1919 (2), 351 Palmetto Drive, Pasadena 2, California

Segrè, Emilio, 1952 (3), Department of Physics, University of California, Berkeley 4, California

Seitz, Frederick, 1951 (3), Department of Physics, University of Illinois, Urbana, Illinois

Serber, Robert, 1952 (3), Department of Physics, Columbia University, New York 27, New York

Shaffer, Philip Anderson, 1928 (14), School of Medicine, Washington University, Euclid Avenue and Kingshighway, St. Louis 10, Missouri

Shannon, Claude Elwood, 1956 (1), Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Shapiro, Harry **L**ionel, 1949 (11), American Museum of Natural History, Central Park West at 79th Street, New York 24, New York

Shapley, Harlow, 1924 (2), Sharon Cross Road, Peterboro, New Hampshire

Shedlovsky, Theodore, 1953 (5), Rockefeller Institute, New York 21, New York

Sheehan, John Clark, 1957 (5), Department of Chemistry, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Shemin, David, 1958 (14), Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York

Sherwood, Thomas Kilgore, 1958 (4), Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Shockley, William, 1951 (4), Shockley Semiconductor Laboratory, 391 South San Antonio Road, Mountain View, California

Shope, Richard Edwin, 1940 (10), Rockefeller Institute, New York 21, New York

Simpson, George **G**aylord, 1941 (6), American Museum of Natural History, 77th Street and Central Park West, New York 24, New York

Sinnott, Edmund Ware, 1936 (7), Osborn Botanical Laboratory, Yale University, New Haven, Connecticut

Skinner, Burrhus **F**rederic, 1950 (12), Memorial Hall, Harvard University, Cambridge 38, Massachusetts

Skoog, Folke Karl, 1956 (7), Department of Botany, Birge Hall, University of Wisconsin, Madison 6, Wisconsin

Slater, John Clarke, 1932 (3), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Slepian, Joseph, 1941 (4), 1115 Lancaster Street, Pittsburgh 18, Pennsylvania

Slichter, Louis Byrne, 1944 (13), Institute of Geophysics, University of California, Los Angeles 24, California

Slipher, Vesto **M**elvin, 1921 (2), Lowell Observatory, Flagstaff, Arizona

Smadel, Joseph Edwin, 1957 (10), National Institutes of Health, Bethesda 14, Maryland

Smith, Cyril Stanley, 1957 (4), Institute for the Study of Metals, University of Chicago, Chicago 37, Illinois

Smith, Gilbert Morgan, 1948 (7), Department of Biology, Stanford University, Stanford, California

Smith, Homer William, 1945 (9), 550 First Avenue, New York 16, New York

Smith, Lee Irvin, 1944 (5), School of Chemistry, University of Minnesota, Minneapolis 14, Minnesota

Smith, Paul Althaus, 1947 (1), Department of Mathematics, Columbia University, New York 27, New York

Smith, Philip Edward, 1939 (8), Department of Anatomy, Stanford University, Stanford, California

Smyth, Charles Phelps, 1955 (5), Department of Chemistry, Princeton University, Princeton, New Jersey

Snell, Esmond Emerson, 1955 (14), Department of Biochemistry, University of California, Berkeley 4, California

Soderberg, Carl Richard, 1947 (4), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Sonneborn, Tracy Morton, 1946 (8), 220 Jordan Hall, Indiana University, Bloomington, Indiana

Spedding, Frank Harold, 1952 (5), Box 14-A, Station A, Institute for Atomic Research, Iowa State College, Ames, Iowa

Spence, Kenneth Wartinbe, 1955 (12), Department of Psychology, State University of Iowa. Iowa City, Iowa

Spier, Leslie, 1946 (11), P. O. Box 880, Santa Cruz, California

Spitzer, Lyman, Jr., 1952 (2), Princeton University Observatory, 14 Prospect Avenue, Princeton, New Jersey

Stakman, Elvin Charles, 1934 (7), Institute of Agriculture, University of Minnesota, St. Paul 1, Minnesota

Stanley, Wendell Meredith, 1941 (14), Virus Laboratory, University of California, Berkeley 4, California

Stebbins, George Ledyard, 1952 (7), Department of Genetics, University of California, Davis, California

Stebbins, Joel, 1920 (2), 69 University Drive, Menlo Park, California

Steenrod, Norman Earl, 1956 (1), Fine Hall, Princeton University, Princeton, New Jersey

Stern, Curt, 1948 (8), Department of Zoology, University of California, Berkeley 4, California

Stern, Otto, 1945 (3), 759 Cragmont Avenue, Berkeley 8, California

Stevens, Stanley Smith, 1946 (12), Memorial Hall, Harvard University, Cambridge 38, Massachusetts

Steward, Julian H., 1954 (11), Department of Sociology and Anthropology, 137 Davenport Hall, University of Illinois, Urbana, Illinois

Stockmayer, Walter Hugo, 1956 (5), Department of Chemistry, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Stone, Marshall Harvey, 1938 (1), 303 Eckhart Hall, University of Chicago, Chicago 37, Illinois

Stratton, Julius Adams, 1950 (4), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Street, Jabez Curry, 1953 (3), Jefferson Physical Laboratory, Harvard University, Cambridge 38, Massachusetts

Struve, Otto, 1937 (2), Department of Astronomy, University of California, Berkeley 4, California

Sturtevant, Alfred Henry, 1930 (8), California Institute of Technology, Pasadena 4, California

Suits, Chauncey Guy, 1946 (4), General Electric Research Laboratory, The Knolls, Schenectady, New York

Szent-Györgyi, Albert, 1956 (14), Institute for Muscle Research, P. O. Box 187, Woods Hole, Massachusetts

Taliaferro, William Hay, 1940 (10), University of Chicago, 5724 Ellis Avenue, Chicago 37, Illinois

Tatum, Edward Lawrie, 1952 (14), Rockefeller Institute, New York 21, New York

Teller, Edward, 1948 (3), Radiation Laboratory, University of California, Berkeley 4, California

Terman, Frederick Emmons, 1946 (4), Stanford University, Stanford, California

Thimann, Kenneth Vivian, 1948 (7), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Thomas, Charles Allen, 1948 (4), Monsanto Chemical Company, P. O. Box 526, St. Louis 3, Missouri

Thomas, Llewellyn Hilleth, 1958 (3), Watson Scientific Computing Laboratory, 612 West 116th Street, New York 27, New York

Thomas, Tracy Yerkes, 1941 (1), Department of Mathematics, Indiana University, Bloomington, Indiana

Thompson, Thomas Gordon, 1951 (13), Department of Oceanography, University of Washington, Seattle 5, Washington

Tillett, William Smith, 1951 (10), New York University College of Medicine, 550 First Avenue, New York 16, New York

Timoshenko, Stephen Prokop, 1940 (4), Room 262, Engineering Building, Stanford University, Stanford, California

Tishler, Max, 1953 (5), Merck & Co., Inc., Rahway, New Jersey

Tolman, Edward Chace, 1937 (12), Department of Psychology, University of California, Berkeley 4, California

Townes, Charles Hard, 1956 (3), Department of Physics, Columbia University, New York 27, New York

Turner, Francis John, 1956 (6), Department of Geology, University of California, Berkeley 4, California

Tuve, Merle Antony, 1946 (3), Department of Terrestrial Magnetism, Carnegie Institution of Washington, 5241 Broad Branch Road, N. W., Washington 15, D. C.

Twitty, Victor Chandler, 1950 (8), Department of Biological Sciences, Stanford University, Stanford, California

Uhlenbeck, George Eugene, 1955 (3), Department of Physics, University of Michigan, Ann Arbor, Michigan

Urey, Harold Clayton, 1935 (5), University of California, La Jolla, California

Van Niel, Cornelis Bernardus, 1945 (7), Hopkins Marine Station of Stanford University, Pacific Grove, California

Van Slyke, Donald Dexter, 1921 (14), Brookhaven National Laboratory, Upton, Long Island, New York

Van Vleck, John Hasbrouck, 1935 (3), Lyman Laboratory of Physics, Harvard University, Cambridge 38, Massachusetts

Veblen, Oswald, 1919 (1), The Institute for Advanced Study, Princeton, New Jersey

Verhoogen, John, 1956 (13), Department of Geology, University of Califorina, Berkeley 4, California

Vestine, Ernest Harry, 1954 (13), Rand Corporation, 1700 Main Street, Santa Monica, California

Vickery, Hubert Bradford, 1943 (14), Connecticut Agricultural Experiment Station, New Haven 4, Connecticut

Villard, Oswald Garrison, Jr., 1958 (13), Radio Propagation Laboratory, Stanford University, Stanford, California

Visscher, Maurice Bolks, 1956 (9), Department of Physiology, University of Minnesota, Minneapolis 14, Minnesota

von **Békésy, Georg**, 1956 (12), Psycho-Acoustic Laboratory, Memorial Hall, Harvard University, Cambridge 38, Massachusetts

von Kármán, Theodore, 1938 (4), 1501 South Marengo Avenue, Pasadena 5, California

Waksman, Selman Abraham, 1942 (7), Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey

Wald, George, 1950 (14), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Walker, John Charles, 1945 (7), 206 Horticulture Building, University of Wisconsin, Madison 6, Wisconsin

Walsh, Joseph Leonard, 1936 (1), Widener 474, Harvard University, Cambridge 38, Massachusetts

Warner, John Christian, 1956 (5), Carnegie Institute of Technology, Pittsburgh 13, Pennsylvania

Webster, David Locke, 1923 (3), 1830 Cowper Street, Palo Alto, California

Weiss, Paul Alfred, 1947 (8), Rockefeller Institute, New York 21, New York

Weisskopf, Victor Frederick, 1952 (3), Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Went, Frits Warmolt, 1947 (7), Missouri Botanical Garden, St. Louis 10, Missouri

Werkman, Chester Hamlin, 1946 (14), Department of Bacteriology, Iowa State College, Ames, Iowa

Westheimer, Frank Henry, 1954 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Wetmore, Alexander, 1945 (8), Smithsonian Institution, Washington 25, D. C.

Wetmore, Ralph Hartley, 1954 (7), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Wever, Ernest Glen, 1940 (12), 110 Eno Hall, Princeton University, Princeton, New Jersey

Wheeler, John Archibald, 1952 (3), Palmer Physical Laboratory, Princeton University, Princeton, New Jersey

Whipple, George Hoyt, 1929 (10), School of Medicine and Dentistry, University of Rochester, 260 Crittenden Boulevard, Rochester 20, New York

Whitford, Albert Edward, 1954 (2), Lick Observatory, Mt. Hamilton, California

Whitney, Hassler, 1945 (1), The Institute for Advanced Study, Princeton, New Jersey

Whyburn, Gordon Thomas, 1951 (1), Department of Mathematics, University of Virginia, Charlottesville, Virginia

Wiggers, Carl John, 1951 (9), 1360 Cleveland Heights Boulevard, Cleveland Heights 21, Ohio

Wigner, Eugene Paul, 1945 (3), 8 Ober Road, Princeton, New Jersey

Williams, Howel, 1950 (6), Department of Geology, University of California, Berkeley 4, California

Williams, John Warren, 1952 (5), Department of Chemistry, University of Wisconsin, Madison 6, Wisconsin

Williams, Robert R., 1945 (5), 297 Summit Avenue, Summit, New Jersey

Williams, Robley Cook, 1955 (14), Virus Laboratory, University of California, Berkeley 4, California

Williams, Roger John, 1946 (5), Clayton Foundation, Biochemical Institute, University of Texas, Austin 12, Texas

Willier, Benjamin Harrison, 1945 (8), Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

Wilson, David **Wright,** 1955 (14), Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania

Wilson, Edgar **Bright, Jr.,** 1947 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Wilson, Edwin Bidwell, 1919 (3), 42 Brington Road, Brookline 46, Massachusetts

Wilson, Perry William, 1955 (7), Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin

Wilson, Ralph Elmer, 1950 (2), 605 Acacia Avenue, Corona del Mar, California

Wilson, Robert Erastus, 1947 (4), 1500 Lake Shore Drive, Chicago 10, Illinois

Wilson, Robert Rathbun, 1957 (3), Laboratory of Nuclear Studies, Cornell University, Ithaca, New York

Winstein, Saul, 1955 (5), Department of Chemistry, University of California, Los Angeles 24, California

Wintersteiner, Oskar, 1950 (14), Squibb Institute for Medical Research, New Brunswick, New Jersey

Wolfrom, Melville Lawrence, 1950 (5), Department of Chemistry, The Ohio State University, Columbus 10, Ohio

Wood, Harland Goff, 1953 (14), Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland 6, Ohio

Woodring, Wendell Phillips, 1946 (6), United States Geological Survey, Washington 25, D. C.

Woodward, Robert Burns, 1953 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Woodworth, Robert Sessions, 1921 (12), 400 West 119th Street, New York 27, New York

Woolley, Dilworth Wayne, 1952 (14), Rockefeller Institute, New York 21, New York

Wright, Sewall Green, 1934 (8), Department of Genetics, University of Wisconsin, Madison 6, Wisconsin

Wright, William Hammond, 1922 (2), 60 North Keeble Avenue, San Jose 26, California

Wu, Chien-Shiung, 1958 (3), Department of Physics, Columbia University, New York 27, New York

Wulf, Oliver Reynolds, 1949 (13), United States Weather Bureau, Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

Wyckoff, Ralph Walter Graystone, 1949 (5), Laboratory of Physical Biology, NIAMD, National Institutes of Health, Bethesda 14, Maryland

Yoder, Hatten Schuyler, Jr., 1958 (6), Geophysical Laboratory, Carnegie Institution of Washington, 2801 Upton Street, N. W., Washington 8, D. C.

Yost, Don Merlin Lee, 1944 (5), California Institute of Technology, Pasadena 4, California

Young, William Gould, 1951 (5), Department of Chemistry, University of California, Los Angeles 24, California

Zacharias, Jerrold Reinach, 1957 (3), Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Zachariasen, Frederik William Houlder, 1949 (3), Department of Physics, University of Chicago, Chicago 37, Illinois

Zariski, Oscar, 1944 (1), Department of Mathematics, Harvard University, 2 Divinity Avenue, Cambridge 38, Massachusetts

Zimm, Bruno Hasbrouck, 1958 (5), General Electric Research Laboratory, Schenectady, New York

Zinn, Walter Henry, 1956 (4), General Nuclear Engineering Corporation, P. O. Box 245, Dunedin, Florida

Zworykin, Vladimir Kosma, 1943 (4), RCA Laboratories Division, David Sarnoff Research Center, Princeton, New Jersey

Number of Members July 1, 1958: 580.

MEMBERS EMERITI

Albright, Fuller, 1952 (10), 271 Goddard Avenue, Brookline 46, Massachusetts

Anderson, John August, 1928 (2), P. O. Box 332, Pasadena 17, California

Bailey, Irving Widmer, 1929 (7), Harvard University Herbarium, 22 Divinity Avenue, Cambridge 38, Massachusetts

Coble, Arthur Byron, 1924 (1), Lykens Hotel, Lykens, Pennsylvania

Coolidge, William David, 1925 (3), 1480 Lenox Road, Schenectady 8, New York

DuBois, Eugene Floyd, 1933 (9), 200 East End Avenue, New York 28, New York

Saunders, Frederick Albert, 1925 (3), South Hadley, Massachusetts

Schultz, Adolph Hans, 1939 (11), Anthropologisches Institut, Sempersteig 3, Zurich, Switzerland

Stadie, William Christopher, 1945 (14), 817 Maloney Clinic Building, 36th and Spruce Streets, Philadelphia 4, Pennsylvania

Tyzzer, Ernest Edward, 1942 (10), 175 Water Street, Wakefield, Massachusetts

Vandiver, Harry Shultz, 1934 (1), Box 7881, University of Texas, Austin 12, Texas

Number of Members Emeriti July 1, 1958: 11.

FOREIGN ASSOCIATES

The number in parentheses following the year of election indicates association within the sections of the National Academy of Sciences.

Adrian of **Cambridge,** Edgar Douglas, Baron, 1941 (9), Trinity College, Cambridge, England

Alexandroff, Paul A., 1947 (1), Mathematical Institute of the Academy of Sciences of the U.S.S.R., Bolshaya Kalushskaya 19, Moscow, U.S.S.R.

Bailey, Sir Edward, 1944 (6), 76 Hampstead Way, London, N. W. 11, England

Bartlett, Sir Frederic Charles, 1947 (12), 161 Huntingdon Road, Cambridge, England

Best, Charles Herbert, 1950 (9), Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

Bjerrum, Niels, 1952 (5), The Royal Veterinary and Agricultural College, Copenhagen, Denmark

Bohr, Niels, 1925 (3), Institute for Theoretical Physics, Blegdamsvej 15, Copenhagen, Denmark

Bordet, Jules, 1935 (10), Pasteur Institute, Rue du Remorquer, 28, Brussels, Belgium

Born, Max, 1955 (3), Marcard Strasse 4, Bad Pyrmont, Germany

Bragg, Sir William **Lawrence,** 1945 (3), The Royal Institution, 21 Albemarle Street, London, W. 1, England

de Broglie, Prince Louis, 1948 (3), 94 Rue Perronet, Neuilly-sur-Seine, France

Burnet, Sir Macfarlane, 1954 (10), The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Caso, Alfonso, 1943 (11), Av. Sudderman No. 312, Col. Chapultepec-Morales, Mexico 5, D. F.

Chapman, Sydney, 1946 (13), High Altitude Observatory, Boulder, Colorado, U. S. A.

Dale, Sir Henry Hallett, 1940 (9), The Wellcome Trust, 52 Queen Anne Street, London, W.1, England

Dirac, Paul Adrien Maurice, 1949 (1), Department of Mathematics, St. John's College, Cambridge, England

Eskola, Pentti Eelis, 1951 (6), Helsinki University, Snellmanink. 5, Helsinki, Finland

Fisher, Sir Ronald Aylmer, 1948 (8), Department of Genetics, University of Cambridge, 44 Storey's Way, Cambridge, England

von Frisch, Karl, 1951 (8), The Zoological Institute, University of Munich, Munich, Germany

Geijer, Per, 1958 (6), Agnevaegen 5, Djursholm I, Sweden

Gregory, Frederick Gugenheim, 1956 (7), Imperial College of Science and Technology, London, S.W. 7, England

Hadamard, Jacques, 1926 (1), 12 Rue Emile Faguet, Paris XIV, France

Hill, Archibald Vivian, 1941 (9), 16 Bishopswood Road, Highgate, London, N.6, England

Hopf, Heinz, 1957 (1), Swiss Federal Institute of Technology, Alte Landstrasse 37, Zurich, Switzerland

Houssay, Bernardo Alberto, 1940 (9), Viamonte 2790, Buenos Aires, Argentina

Jeffreys, Sir Harold, 1945 (13), St. John's College, Cambridge, England

Jones, Sir Harold Spencer, 1943 (2), 40 Hesper Mews, London, S.W. 5, England

Kapitza, Peter Leonidovich, 1946 (3), Institute for Physical Problems, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

Karrer, Paul, 1945 (5), University of Zurich, Zurich, Switzerland

Kihara, Hitoshi, 1958 (7), National Institute of Genetics, Misima, Japan

Krishnan, Sir Kariamanikkam Srinivasa, 1956 (3), National Physical Laboratory of India, Hillside Road, New Delhi 12, India

von Laue, Max, 1958 (3), Fritz Haber Institute of The Max Planck Society, Faradayweg 4, Berlin-Dahlem, Germany

Levi, Giuseppe, 1940 (8), Instituto di Anatomia Umana, Corso Massimo D'Azeglio, 52, Turin, Italy

Lim, Robert K. S., 1942 (9), The Miles-Ames Research Laboratory, Elkhart, Indiana, U. S. A.

Lindblad, Bertil, 1955 (2), Stockholm Observatory, Saltsjöbaden, Sweden

Linderstrøm-Lang, Kaj Ulrik, 1947 (5), Chemical Division, Carlsberg Laboratory, Copenhagen, Denmark

Lwoff, André, 1955 (9), Institut Pasteur, Paris XV, France

Michotte, Albert Edouard (Baron Michotte van den Berck), 1956 (12), University of Louvain, Louvain, Belgium

Mott, Nevill Francis, 1957 (3), University of Cambridge, Cavendish Laboratory, Free School Lane, Cambridge, England

Oort, Jan Hendrik, 1953 (2), Observatory of Leiden, Leiden, The Netherlands

Penfield, Wilder, 1953 (10), Montreal Neurological Institute, 3801 University Street, Montreal 2, Canada

Pérès, Joseph Jean Camille, 1956 (1), University of Paris, Paris V, France

Pieron, Henri, 1949 (12), Institute of Psychology, University of Paris, Paris, France

Portevin, Albert M. G. R., 1954 (4), 21, Boulevard de Beauséjour, Paris XVI, France

Reichstein, Tadeus, 1952 (5), Organisch-chemische Anstalt, St. Johanns-Ring 19, Basel, Switzerland

Renner, Otto, 1954 (7), The Botanical Institute, University of Munich, Munich, Germany

Robinson, Sir Robert, 1934 (5), 170 Piccadilly, London, W. 1, England

Ruzicka, Leopold, 1944 (5), Department of Organic Chemistry, Institute of Technology, Zurich, Switzerland

Southwell, Sir Richard Vynne, 1943 (4), The Old House, Trumpington, Cambridge, England

Steacie, Edgar William Richard, 1957 (5), National Research Council, Ottawa, Canada

Svedberg, The, 1945 (5), Fysikalisk-Kemiska Institutionen, Uppsala University, Uppsala, Sweden

Taylor, Sir Geoffrey Ingram, 1945 (1), Trinity College, Cambridge, England

Theorell, Axel **Hugo,** 1957 (14), Nobel Institute of Medicine, Solnavagen 1, Stockholm 60, Sweden

Tiselius, Arne W. K., 1949 (14), Institute of Biochemistry, Uppsala University, Uppsala, Sweden

Todd, Sir Alexander Robertus, 1955 (5), University of Cambridge, University Chemical Laboratory, Pembroke Street, Cambridge, England

Vallée-Poussin, C. de la, 1929 (1), 42, Avenue du Houx, Boitsfort, Belgium

Vening Meinesz, Felix Andries, 1939 (13), Potgieterlaan 5, Amersfoort, The Netherlands

Watson, D. M. S., 1938 (8), University College, Gower Street, London, W.C.1, England

Winge, Öjvind, 1949 (14), Department of Physiology, Carlsberg Laboratory, Copenhagen (Valby), Denmark

Yukawa, Hideki, 1949 (3), Yukawa Hall, Kyoto University, Kyoto, Japan

Number of Foreign Associates July 1, 1958: 60.

SECTIONS

(1) *Mathematics*—32 members

Albert, A. A., *Chairman* (1961)	Eisenhart, L. P.	Murnaghan, F. D.
Ahlfors, L. V.	Evans, G. C.	Shannon, C. E.
Alexander, J. W.	Gödel, Kurt	Smith, Paul A.
Artin, E.	Hille, Einar	Steenrod, N. E.
Bell, E. T.	Jacobson, Nathan	Stone, M. H.
Bochner, S.	Lefschetz, Solomon	Thomas, T. Y.
Brauer, R. D.	Mac Lane, Saunders	Veblen, Oswald
Courant, R.	McShane, E. J.	Walsh, J. L.
Doob, J. L.	Montgomery, Deane	Whitney, Hassler
Douglas, Jesse	Moore, R. L.	Whyburn, G. T.
	Morse, Marston	Zariski, O.

Foreign Associates

Alexandroff, P. A.	Hopf, Heinz	Taylor, Sir Geoffrey
Dirac, P. A. M.	Pérès, Joseph	Vallée-Poussin, C. de la
Hadamard, Jacques		

(2) *Astronomy*—29 members

Brouwer, Dirk, *Chairman* (1959)	Clemence, G. M.	Menzel, D. H.
Abbot, C. G.	Goldberg, Leo	Merrill, P. W.
Babcock, H. D.	Greenstein, J. L.	Mitchell, S. A.
Babcock, H. W.	Joy, A. H.	Morgan, W. W.
Bowen, I. S.	Kuiper, G. P.	Nicholson, S. B.
Chandrasekhar, S.	Mayall, N. U.	Ross, F. E.
	McMath, R. R.	Schwarzschild, M.

Seares, F. H.
Shapley, Harlow
Slipher, V. M.
Spitzer, Lyman, Jr.
Stebbins, Joel
Struve, Otto
Whitford, A. E.
Wilson, Ralph E.
Wright, W. H.

Foreign Associates

Jones, Sir H. Spencer
Lindblad, Bertil
Oort, Jan Hendrik

(3) *Physics*—84 members

Loomis, F. W., *Chairman* (1960)
Allison, S. K.
Alvarez, L. W.
Anderson, C. D.
Bacher, R. F.
Bainbridge, K. T.
Bardeen, John
Beams, J. W.
Bethe, H. A.
Birge, R. T.
Bloch, Felix
Bradbury, N. E.
Breit, Gregory
Bridgman, P. W.
Brillouin, Leon
Brode, R. B.
Coblentz, W. W.
Compton, A. H.
Condon, E. U.
Dennison, D. M.
Deutsch, M.
DuBridge, L. A.
DuMond, J. W. M.
Dunning, J. R.
Epstein, P. S.
Feynman, R. P.
Fowler, W. A.
Franck, James
Gamow, George
Goldhaber, M.
Goudsmit, S. A.
Herb, R. G.
Hofstadter, R.
Houston, W. V.
Hull, A. W.
Kemble, E. C.
Kent, R. H.
Kerst, D. W.
Kittel, C.
Kusch, P.
Lamb, W. E., Jr.
Land, E. H.
Lauritsen, C. C.
Lawrence, E. O.
Marshak, R. E.
Mason, Max
Mayer, M. G.
McMillan, E. M.
Meggers, W. F.
Morse, P. M.
Mulliken, R. S.
Nier, A. O. C.
O'Brien, Brian
Oppenheimer, J. R.
Panofsky, W. K. H.
Pegram, G. B.
Purcell, E. M.
Rabi, I. I.
Ramsey, N. F.
Robertson, H. P.
Rossi, Bruno
Schiff, L. I.
Schwinger, Julian
Segrè, E.
Seitz, Frederick
Serber, R.
Slater, J. C.
Stern, Otto
Street, J. C.
Teller, Edward
Thomas, L. H.
Townes, C. H.
Tuve, M. A.
Uhlenbeck, G. E.
Van Vleck, J. H.
Webster, D. L.
Weisskopf, V. F.
Wheeler, J. A.
Wigner, E. P.
Wilson, Edwin B.
Wilson, Robert R.
Wu, C. S.
Zacharias, J. R.
Zachariasen, W. H.

Foreign Associates

Bohr, Niels
Born, Max
Bragg, Sir Lawrence
de Broglie, Prince Louis
Kapitza, P. L.
Krishnan, Sir K.
von Laue, M.
Mott, N. F.
Yukawa, Hideki

(4) *Engineering*—45 members

Foote, P. D., *Chairman* (1959)
Bain, E. C.
Benedict, Manson
Bode, H. W.
Briggs, L. J.
Buckley, O. E.
Bush, Vannevar
Chipman, John
Cochrane, E. L.
Curme, G. O., Jr.
Den Hartog, J. P.
Draper, C. S.
Dryden, H. L.
Durand, W. F.
Fisk, J. B.
Fletcher, Harvey
Gibbs, W. F.

Gilliland, E. R.
Greenewalt, C. H.
Hoover, Herbert
Hunsaker, J. C.
Jeffries, Zay
Kelly, M. J.
Kettering, C. F.
Lewis, W. K.
Loomis, A. L.
Mehl, R. F.
Murphree, E. V.
Pierce, J. R.
Raymond, A. E.
Savage, J. L.
Sherwood, T. K.
Shockley, W.
Slepian, Joseph
Smith, C. S.
Soderberg, C. R.
Stratton, J. A.
Suits, C. G.
Terman, F. E.
Thomas, C. A.
Timoshenko, Stephen
von Kármán, T.
Wilson, Robert E.
Zinn, W. H.
Zworykin, V. K.

Foreign Associates

Portevin, Albert M. G. R.
Southwell, Sir Richard V.

(5) *Chemistry*—85 members

Cope, A. C., *Chairman* (1959)
Adams, Roger
Badger, R. M.
Bartlett, P. D.
Bolton, E. K.
Brode, W. R.
Brown, H. C.
Calvin, Melvin
Conant, J. B.
Craig, L. C.
Crawford, Bryce, Jr.
Daniels, Farrington
Debye, Peter
Doty, Paul
Elderfield, R. C.
Emmett, P. H.
Eyring, Henry
Fieser, L. F.
Flory, P. J.
Folkers, Karl
Fuoss, R. M.
Fuson, R. C.
Gates, M.
Giauque, W. F.
Gilman, Henry
Hammett, L. P.
Harned, H. S.
Hauser, C. R.
Hendricks, S. B.
Hildebrand, J. H.
Hirschfelder, J. A.
Hornig, D. F.
Jacobs, W. A.
Johnson, J. R.
Johnson, W. S.
Keyes, F. G.
Kimball, G. E.
Kirkwood, J. G.
Kistiakowsky, G. B.
Kolthoff, I. M.
Kraus, C. A.
La Mer, V. K.
Leonard, N. J.
Libby, W. F.
Lind, S. C.
Longsworth, L. G.
Lucas, H. J.
MacInnes, D. A.
Marvel, C. S.
Mayer, J. E.
McElvain, S. M.
Mees, C. E. K.
Newman, M. S.
Niemann, C. G.
Noyes, W. A., Jr.
Onsager, Lars
Pauling, Linus
Pitzer, K. S.
Roberts, John D.
Rodebush, W. H.
Rossini, F. D.
Scatchard, George
Schlesinger, H. I.
Seaborg, G. T.
Shedlovsky, Theodore
Sheehan, J. C.
Smith, L. I.
Smyth, C. P.
Spedding, F. H.
Stockmayer, W. H.
Tishler, Max
Urey, H. C.
Warner, J. C.
Westheimer, F. H.
Williams, J. W.
Williams, Robert R.
Williams, Roger J.
Wilson, E. Bright, Jr.
Winstein, Saul
Wolfrom, M. L.
Woodward, R. B.
Wyckoff, R. W. G.
Yost, D. M.
Young, W. G.
Zimm, B. H.

Foreign Associates

Bjerrum, Niels
Karrer, Paul
Linderstrøm-Lang, K. U.
Reichstein, Tadeus
Robinson, Sir Robert
Ruzicka, Leopold
Steacie, E. W. R.
Svedberg, The
Todd, Sir Alexander

(6) *Geology*—33 members

Woodring, W. P., *Chairman* (1960)
Allen, E. T.
Anderson, C. A.
Birch, Francis
Blackwelder, Eliot
Bradley, W. H.
Bramlette, M. N.
Bucher, W. H.
Buddington, A. F.
Buerger, M. J.
Chaney, R. W.
Cloos, Ernst
Day, A. L.
Dunbar, C. O.
Gilluly, James
Gregory, W. K.
Hess, H. H.
Hewett, D. F.
Hubbert, M. King
Kelley, W. P.
Knopf, Adolph
Larsen, E. S., Jr.
Longwell, C. R.
Lovering, T. S.
Mead, W. J.
Nolan, T. B.
Reeside, J. B., Jr.
Rubey, W. W.
Schairer, J. F.
Simpson, G. G.
Turner, F. J.
Williams, Howel
Yoder, H. S., Jr.

Foreign Associates

Bailey, Sir Edward
Eskola, Pentti
Geijer, Per

(7) *Botany*—43 members

Goddard, D. R., *Chairman* (1959)
Anderson, Edgar
Barker, H. A.
Beadle, G. W.
Blinks, L. R.
Bonner, James
Brink, R. A.
Burkholder, P. R.
Chandler, W. H.
Cleland, R. E.
Couch, J. N.
Delbrück, Max
Dodge, B. O.
Emerson, Robert
Esau, Katherine
Fred, E. B
Hershey, A. D.
Hollaender, A.
Horsfall, J. G.
Jones, D. F.
Kaufmann, B. P.
Kunkel, L. O.
Lederberg, J.
Mangelsdorf, P. C.
McClintock, Barbara
Osterhout, W. J. V.
Raper, K. B.
Rhoades, M. M.
Riker, A. J.
Robbins, W. J.
Sax, Karl
Sinnott, E. W.
Skoog, Folke
Smith, Gilbert M.
Stakman, E. C.
Stebbins, G. L.
Thimann, K. V.
Van Niel, C. B.
Waksman, S. A.
Walker, J. C.
Went, F. W.
Wetmore, R. H.
Wilson, P. W.

Foreign Associates

Gregory, F. G.
Kihara, H.
Renner, Otto

(8) *Zoology and Anatomy*—50 members

Demerec, Milislav, *Chairman* (1961)
Bartelmez, G. W.
Bigelow, H. B.
Bloom, William
Bodenstein, D. H.
Bodian, David
Burns, R. K.
Castle, W. E.
Cleveland, L. R.
Colbert, E. H.
Corner, G. W.
Danforth, C. H.
Dobzhansky, Th.
Dunn, L. C.
Hamburger, Viktor
Harrison, R. G.
Hartman, C. G.
Harvey, E. N.
Haskins, C. P.
Herrick, C. J.
Hisaw, F. L.
Holtfreter, J.
Hubbs, C. L.

Hutchinson, G. E.
Irwin, M. R.
Jacobs, M. H.
Kellogg, Remington
Lewis, W. H.
Mayr, Ernst
Metz, C. W.
Miller, A. H.
Mirsky, A. E.
Muller, H. J.
Nicholas, J. S.
Painter, T. S.
Patterson, J. T.
Petrunkevitch, A.
Riddle, Oscar
Romer, A. S.
Schmitt, F. O.
Schrader, Franz
Smith, Philip E.
Sonneborn, T. M.
Stern, Curt
Sturtevant, A. H.
Twitty, V. C.
Weiss, Paul
Wetmore, Alexander
Willier, B. H.
Wright, Sewall

Foreign Associates

Fisher, Sir Ronald
von Frisch, Karl
Levi, Giuseppe
Watson, D. M. S.

(9) *Physiology*—32 members

Bard, Philip, *Chairman* (1960)
Astwood, E. B.
Aub, Joseph C.
Bronk, Detlev W.
Cole, K. S.
Cournand, André
Davis, Hallowell
Erlanger, Joseph
Evans, H. M.
Fenn, W. O.
Forbes, Alexander
Gasser, H. S.
Gerard, R. W.
Hartline, H. K.
Hastings, A. B.
Landis, E. M.
Lloyd, D. P. C.
Loeb, R. F.
Long, C. N. H.
Lorente de Nó, R.
Magoun, H. W.
Mann, Frank
Marshall, E. K., Jr.
Meek, W. J.
Pitts, R. F.
Redfield, A. C.
Richards, A. N.
Richards, D. W.
Schmidt, C. F.
Smith, Homer W.
Visscher, M. B.
Wiggers, C. J.

Foreign Associates

Lord Adrian
Best, C. H.
Dale, Sir Henry
Hill, A. V.
Houssay, B. A.
Lim, Robert K. S.
Lwoff, André

(10) *Pathology and Microbiology*—40 members

Paul, John R., *Chairman* (1960)
Armstrong, Charles
Bailey, P.
Blalock, Alfred
Cannon, P. R.
Castle, W. B.
Coggeshall, L. T.
Cole, Rufus
Dalldorf, Gilbert
Dingle, J. H.
Dochez, A. R.
Dragstedt, L. R.
Dubos, R. J.
Enders, J. F.
Francis, Thomas, Jr.
Gamble, J. L.
Goebel, W. F.
Goodpasture, E. W.
Heidelberger, Michael
Horsfall, F. L., Jr.
Huggins, C. B.
Little, C. C.
Loeb, Leo
Long, E. R.
MacLeod, C. M.
Maxcy, K. F.
McMaster, P. D.
Meyer, K. F.
Miller, C. P.
Opie, E. L.
Rich, A. R.
Rivers, T. M.
Robertson, O. H.
Rous, Peyton
Sabin, Albert B.
Shope, R. E.
Smadel, J. E.
Taliaferro, W. H.
Tillett, W. S.
Whipple, G. H.

Foreign Associates

Bordet, Jules
Burnet, Sir Macfarlane
Penfield, Wilder

(11) *Anthropology*—11 members

Kluckhohn, Clyde, *Chairman* (1960)
Albright, W. F.
Coon, C. S.
Haury, Emil W.
Kidder, A. V.
Kroeber, A. L.
Lothrop, S. K.
Movius, H. L., Jr.
Shapiro, H. L.
Spier, Leslie
Steward, J. H.

Foreign Associate

Caso, Alfonso

(12) *Psychology*—23 members

Beach, F. A., *Chairman* (1959)
Boring, E. G.
Carmichael, Leonard
Gesell, Arnold
Graham, C. H.
Guilford, J. P.
Harlow, H. F.
Hilgard, E. R.
Klüver, Heinrich
Köhler, Wolfgang
Lashley, K. S.
Lindsley, D. B.
Miles, W. R.
Miller, N. E.
Pillsbury, W. B.
Richter, C. P.
Skinner, B. F.
Spence, K. W.
Stevens, S. S.
Tolman, E. C.
v. Békésy, Georg
Wever, E. G.
Woodworth, R. S.

Foreign Associates

Bartlett, Sir Frederic
Michotte, Albert E.
Pieron, Henri

(13) *Geophysics*—28 members

Byerly, Perry, *Chairman* (1960)
Adams, L. H.
Benioff, Hugo
Berkner, L. V.
Bjerknes, J.
Brown, Harrison
Byers, H. R.
Eckart, Carl
Elsasser, W. M.
Ewing, Maurice
Griggs, D. T.
Gunn, Ross
Gutenberg, Beno
Iselin, C. O'D.
Kaplan, Joseph
Lambert, W. D.
Munk, W. H.
Pekeris, C. L.
Piggot, C. S.
Press, Frank
Reichelderfer, F. W.
Revelle, Roger
Slichter, L. B.
Thompson, T. G.
Verhoogen, John
Vestine, E. H.
Villard, O. G., Jr.
Wulf, O. R.

Foreign Associates

Chapman, Sydney
Jeffreys, Sir Harold
Vening Meinesz, F. A

(14) *Biochemistry*—45 members

du Vigneaud, V., *Chairman* (1961)
Anderson, R. J.
Ball, E. G.
Balls, A. K.
Bloch, K. E.
Carter, H. E.
Chance, Britton
Clark, W. M.
Clarke, H. T.
Cori, Carl F.
Doisy, E. A.
Edsall, J. T.
Elvehjem, C. A.
Fischer, H. O. L.
Fruton, J. S.
Hassid, W. Z.
Kendall, E. C.

King, C. G.
Kornberg, A.
Lardy, Henry
Lehninger, A. L.
Link, K. P.
Lipmann, Fritz
Maynard, L. A.
McCollum, E. V.
Northrop, J. H.
Ochoa, Severo
Oncley, J. L.
Rittenberg, David
Rose, W. C.
Shaffer, P. A.
Shemin, David
Snell, E. E.
Stanley, W. M.
Szent-Györgyi, A.
Tatum, E. L.
Van Slyke, D. D.
Vickery, H. B.
Wald, George
Werkman, C. H.
Williams, R. C.
Wilson, D. W.
Wintersteiner, O.
Wood, H. G.
Woolley, D. W.

Foreign Associates

Theorell, Hugo
Tiselius, Arne W. K.
Winge, Öjvind

GEOGRAPHICAL LISTING of ACADEMY MEMBERS

Arizona

Haury, Emil W.
Slipher, V. M.

California

Adams, L. H.
Alvarez, L. W.
Anderson, C. D.
Anderson, J. A.
Babcock, H. D.
Babcock, H. W.
Bacher, R. F.
Badger, R. M.
Balls, A. K.
Barker, H. A.
Beach, F. A.
Beadle, G. W.
Bell, E. T.
Benioff, Hugo
Birge, R. T.
Bjerknes, J.
Blackwelder, Eliot
Blinks, L. R.
Bloch, Felix
Bonner, James
Bowen, I. S.
Bramlette, M. N.
Brode, R. B.
Brown, Harrison
Byerly, Perry
Calvin, Melvin
Castle, W. E.
Chandler, W. H.
Chaney, R. W.
Danforth, C. H.
Delbrück, Max
DuBridge, L. A.
DuMond, J. W. M.
Eckart, Carl
Elsasser, W. M.
Epstein, P. S.
Esau, Katherine
Evans, G. C.
Evans, H. M.
Feynman, R. P.
Fischer, H. O. L.
Fowler, W. A.
Giauque, W. F.
Greenstein, J. L.
Griggs, D. T.
Guilford, J. P.
Gutenberg, Beno
Hassid, W. Z.
Hewett, D. F.
Hildebrand, J. H.
Hilgard, E. R.
Hofstadter, R.
Hubbs, C. L.
Joy, A. H.
Kaplan, Joseph
Kelley, W. P.
Kerst, D. W.
Kittel, C.
Knopf, Adolph
Kroeber, A. L.
Lauritsen, C. C.
Lawrence, E. O.
Lindsley, D. B.
Longwell, C. R.
Lucas, H. J.
Magoun, H. W.
Mason, Max
Mayall, N. U.
McMillan, E. M.
Merrill, P. W.
Meyer, K. F.
Miller, A. H.
Munk, W. H
Nicholson, S. B.
Niemann, Carl
Northrop, J. H.
Panofsky, W. K. H.
Pauling, Linus
Pitzer, K. S.
Press, Frank
Raymond, A. E.
Revelle, Roger
Roberts, John D.
Robertson, H. P.
Robertson, O. H.
Ross, F. E.
Schiff, L. I.
Seaborg, G. T.
Seares, F. H.
Segrè, Emilio
Shannon, C. E.
Shockley, W.
Slichter, L. B.

Smith, G. M.
Smith, P. E.
Snell, E. E.
Spier, Leslie
Stanley, W. M.
Stebbins, G. L.
Stebbins, Joel
Stern, Curt
Stern, Otto
Struve, Otto
Sturtevant, A. H.
Teller, Edward
Terman, F. E.
Timoshenko, S. P.
Tolman, E. C.
Turner, F. J.
Twitty, V. C.
Urey, H. C.
Van Niel, C. B.
Verhoogen, John
Vestine, E. H.
Villard, O. G., Jr.
von Kármán, T.
Webster, D. L.
Whitford, A. E.
Williams, Howel
Williams, R. C.
Wilson, Ralph E.
Winstein, Saul
Wright, W. H.
Wulf, O. R.
Yost, D. M.
Young, W. G.

Colorado

*Chapman, Sydney
Gamow, George
Gilluly, James
Lovering, T. S.
Savage, J. L.

Connecticut

Anderson, R. J.
Breit, G.
Brouwer, Dirk
Clarke, H. T.
Dunbar, C. O.
Fruton, J. S.
Fuoss, R. M.
Gesell, Arnold
Harned, H. S.
Harrison, R. G.
Hille, Einar
Horsfall, J. G.
Hutchinson, G. E.
Jacobson, Nathan
Jones, D. F.
Kirkwood, J. G.
Lambert, W. D.
Long, C. N. H.
Miles, W. R.
Miller, N. E.
Nicholas, J. S.
Onsager, Lars
Paul, J. R.
Petrunkevitch, A.
Sinnott, E. W.
Vickery, H. B.

Delaware

Bolton, E. K.
Greenewalt, C. H.

District of Columbia

Abbot, C. G.
Anderson, C. A.
Bradley, W. H.
Briggs, L. J.
Brode, W. R.
Carmichael, Leonard
Clemence, G. M.
Coblentz, W. W.
Dryden, H. L.
Foote, P. D.
Goodpasture, E. W.
Gunn, Ross
Haskins, C. P.
Kellogg, Remington
Larsen, E. S., Jr.
Libby, W. F.
Meggers, W. F.
Nolan, T. B.
Piggot, C. S.
Reichelderfer, F. W.
Rubey, W. W.
Schairer, J. F.
Tuve, M. A.
Wetmore, Alexander
Woodring, W. P.
Yoder, H. S., Jr.

Florida

Lashley, K. S.
Riddle, Oscar
Zinn, W. H.

Illinois

Adams, Roger
Albert, A. A.
Allison, S. K.
Bailey, P.
Bardeen, John
Bloom, William
Byers, H. R.
Cannon, Paul
Carter, H. E.
Coggeshall, L. T.
Doob, J. L.
Dragstedt, L. R.
Emerson, Robert
Franck, James
Fuson, R. C.
Huggins, Charles
Klüver, Heinrich
Leonard, N. J.
Loomis, F. W.
Mac Lane, Saunders
Marvel, C. S.
Mayer, J. E.
Mayer, M. G.
Miller, C. P.
Mulliken, R. S.
Rhoades, M. M.
Rodebush, W. H.
Rose, W. C.
Schlesinger, H. I.
Seitz, Frederick
Smith, C. S.
Steward, J. H.
Stone, M. H.
Taliaferro, W. H.
Wilson, Robert E.
Zachariasen, W. H.

Indiana

Brown, H. C.
Cleland, R. E.
*Lim, R. K. S.
Muller, H. J.
Sonneborn, T. M.
Thomas, T. Y.

* Foreign Associate.

Iowa

Gilman, Henry
Spedding, F. H.
Spence, K. W.
Werkman, C. H.

Maine

Little, C. C.

Maryland

Albright, W. F.
Armstrong, Charles
Bard, Philip
Blalock, Alfred
Bodenstein, D. H.
Bodian, David
Burns, R. K.
Clark, W. M.
Cloos, Ernst
Cole, K. S.
Day, A. L.
Emmett, P. H.
Hendricks, S. B.
Kent, R. H.
Lehninger, A. L.
Marshall, E. K., Jr.
Maxcy, K. F.
McCollum, E. V.
Murnaghan, F. D.
Reeside, J. B., Jr.
Rich, A. R.
Richter, C. P.
Smadel, J. E.
Willier, B. H.
Wyckoff, R. W. G.

Massachusetts

Ahlfors, L. V.
Albright, Fuller
Allen, E. T.
Astwood, E. B.
Aub, Joseph C.
Bailey, I. W.
Bainbridge, K. T.
Ball, E. G.
Bartlett, P. D.
Benedict, Manson
Bigelow, H. B.
Birch, Francis
Bloch, K. E.
Boring, E. G.
Brauer, R.
Bridgman, P. W.
Buerger, M. J.
Bush, Vannevar
Castle, W. B.
Chipman, John
Cleveland, L. R.
Cochrane, E. L.
Cope, A. C.
Den Hartog, J. P.
Deutsch, M.
Doty, Paul
Draper, C. S.
Edsall, J. T.
Enders, J. F.
Fieser, L. F.
Forbes, Alexander
Gamble, J. L.
Gilliland, E. R.
Hastings, A. B.
Hisaw, F. L.
Hunsaker, J. C.
Iselin, C. O'D.
Jeffries, Zay
Kemble, E. C.
Keyes, F. G.
Kidder, A. V.
Kimball, G. E.
Kistiakowsky, G. B.
Kluckhohn, Clyde
Land, E. H.
Landis, E. M.
Lewis, W. K.
Lothrop, S. K.
Mangelsdorf, P. C.
Mayr, Ernst
Mead, W. J.
Menzel, D. H.
Metz, C. W.
Morse, P. M.
Movius, H. L., Jr.
O'Brien, Brian
Oncley, J. L.
Purcell, E. M.
Ramsey, Norman
Redfield, A. C.
Romer, A. S.
Rossi, B. B.
Saunders, F. A.
Sax, Karl
Scatchard, George
Schmitt, F. O.
Schwinger, Julian
Sheehan, J. C.
Sherwood, T. K.
Skinner, B. F.
Slater, J. C.
Soderberg, C. R.
Stevens, S. S.
Stockmayer, W.
Stratton, J. A.
Street, J. C.
Szent-Györgyi, A.
Thimann, K. V.
Tyzzer, E. E.
Van Vleck, J. H.
v. Békésy, Georg
Wald, George
Walsh, J. L.
Weisskopf, V. F.
Westheimer, F. H.
Wetmore, R. H.
Wilson, E. Bright, Jr.
Wilson, Edwin B.
Woodward, R. B.
Zacharias, J. R.
Zariski, Oscar

Michigan

Dennison, D. M.
Elderfield, R. C.
Francis, Thomas, Jr.
Gerard, R. W.
Goldberg, Leo
Herrick, C. J.
Kettering, C. F.
McMath, R. R.
Pillsbury, W. B.
Uhlenbeck, G. E.

Minnesota

Crawford, Bryce, Jr.
Kolthoff, I. M.
Mann, F. C.
Nier, A. O. C.
Smith, L. I.
Stakman, E. C.
Visscher, M. B.

Missouri

Anderson, Edgar
Compton, A. H.

Condon, E. U.
Cori, C. F.
Davis, Hallowell
Doisy, E. A.
Erlanger, Joseph
Hamburger, Viktor
Kornberg, A.
Loeb, Leo
Shaffer, P. A.
Thomas, C. A.
Went, F. W.

Montana

Bartelmez, G. W.

New Hampshire

Shapley, Harlow

New Jersey

Alexander, J. W.
Bochner, S.
Bode, H. W.
Buckley, O. E.
Buddington, A. F.
Eisenhart, L. P.
Fisk, J. B.
Folkers, Karl
Gödel, Kurt
Hartman, C. G.
Harvey, E. N.
Heidelberger, M.
Hess, H. H.
Hornig, D. F.
Kendall, E. C.
Lefschetz, Solomon
Montgomery, Deane
Morse, Marston
Murphree, E. V.
Oppenheimer, J. R.
Pierce, J. R.
Schwarzschild, M.
Smyth, C. P.
Spitzer, Lyman, Jr.
Steenrod, N. E.
Tishler, Max
Veblen, Oswald
Waksman, S. A.
Wever, E. G.
Wheeler, J. A.
Whitney, Hassler
Wigner, E. P.
Williams, R. R.
Wintersteiner, Oskar
Zworykin, V. K.

New Mexico

Bradbury, N. E.

New York

Berkner, L. V.
Bethe, H. A.
Brillouin, Leon
Bronk, D. W.
Bucher, W. H.
Burkholder, P. R.
Colbert, E. H.
Cole, Rufus
Conant, J. B.
Coolidge, W. D.
Corner, G. W.
Courant, Richard
Cournand, André
Craig, L. C.
Curme, G. O., Jr.
Dalldorf, Gilbert
Debye, Peter
Demerec, Milislav
Dobzhansky, Th.
Dochez, A. R.
Dodge, B. O.
Douglas, Jesse
DuBois, E. F.
Dubos, R. J.
Dunn, L. C.
Dunning, J. R.
Durand, W. F.
du Vigneaud, Vincent
Ewing, Maurice
Fenn, W. O.
Gasser, H. S.
Gates, M.
Gibbs, W. F.
Goebel, W. F.
Goldhaber, M.
Goudsmit, SA.
Graham, C. H.
Gregory, W. K.
Hammett, L. P.
Hartline, H. K.
Hershey, A. D.
Holtfreter, J.
Hoover, Herbert
Horsfall, F. L., Jr.
Hull, A. W.
Jacobs, W. A.
Johnson, J. R.
Kaufmann, B. P.
Kelly, M. J.
King, C. G.
Kunkel, L. O.
Kusch, P.
La Mer, V. K.
Lipmann, Fritz
Lloyd, D. P. C.
Loeb, R. F.
Longsworth, L. G.
Loomis, A. L.
Lorente de Nó, R.
MacInnes, D. A.
Marshak, R. E.
Maynard, L. A.
McClintock, Barbara
McMaster, P. D.
Mirsky, A. E.
Noyes, W. A., Jr.
Ochoa, Severo
Opie, E. L.
Osterhout, W. J. V.
Pegram, G. B.
Pitts, R. F.
Rabi, I. I.
Richards, D. W.
Rittenberg, David
Rivers, T. M.
Robbins, W. J.
Rous, Peyton
Schrader, Franz
Serber, Robert
Shapiro, H. L.
Shedlovsky, Theodore
Shemin, David
Shope, R. E.
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